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Regulación del ciclo celular y el desarrollo de tumores *in vivo* por inhibidores INK4 y Cip/Kip

Tesis Doctoral

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Certifica: que Don Víctor Quereda Sánchez-Alarcos ha realizado bajo su dirección el trabajo de tesis titulado:

Regulación del ciclo celular y el desarrollo de tumores in vivo por inhibidores INK4 y Cip/Kip

Revisado el presente trabajo, considera que reúne todos los méritos necesarios para su presentación y defensa con el fin de optar al grado de Doctor por la Universidad Autónoma de Madrid.

Director,
Marcos Malumbres Martínez

A mis padres

“El azar favorece a la mente preparada”

Alexander Fleming

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Resumen / Summary

La desregulación del ciclo celular es un fenómeno fundamental en el desarrollo del cáncer. El conocimiento de las bases moleculares que llevan a esta desregulación supone por tanto uno de los pilares básicos en el estudio del cáncer. La hiperactivación de las quinasas dependeientes de ciclinas (Cdks) es una de las alteraciones moleculares que aparecen con más frecuencia en las células tumorales. Los inhibidores de Cdks de las familias INK4 y Cip/Kip han sido relacionados con la senescencia celular y supresión tumoral, aunque tampoco se puede descartar un papel oncogénico en determinadas condiciones. Para comprender el papel de estos inhibidores y su efecto sinérgico hemos caracterizado *in vitro* e *in vivo* el efecto producido al combinar la eliminación de $p21^{Cip1}$ y $p27^{Kip1}$ en un fondo genético Cdk4 R24C, que hace a esta proteína insensible a la inhibición por las proteínas de la familia INK4. Hemos podido comprobar como la inactivación conjunta de las vías de señalización de $p21^{Cip1}$ e INK4 cooperan en la supresión de la senescencia celular *in vitro* y en la aparición de tumores in vivo, especialmente los de origen mesenquimal. Según vamos introduciendo mutaciones en los distintos alelos de Cdk4 R24C, $p21^{Cip1}$ y $p27^{Kip1}$, la latencia en la aparición de tumores va disminuyendo y el origen se va restringiendo hacia los de origen endocrino, especialmente los tumores de hipófisis. En colaboración con el grupo de Clara Álvarez de la Universidad de Santiago de Compostela hemos caracterizado un tipo celular específico en este órgano con características de células madres y hemos estudiado la desregulación de estas células progenitoras en los distintos modelos. Finalmente, la inactivación combinada de los inhibidores INK4 (en el modelo Cdk4 R24C) y de $p21^{Cip1}$ y $p27^{Kip1}$ provoca una hipoplasia generalizada probablemente debido al estrés replicativo provocado por la pérdida de los frenos del ciclo celular.

SUMMARY

Cell cycle deregulation is a major feature of cancer cells. Knowing the molecular basis of this deregulation should be, therefore, one of the main objectives in order to understand cancer development. Cdks hyperactivation is one of the molecular alterations more frequently found in tumoral cells. The INK4 and Cip/Kip families of Cdk inhibitors have been related to cellular senescence and tumoral suppression, although an oncogenic role has been also described for Cip/Kip inhibitors. To understand the role of these inhibitors and their synergistic effect we have characterized in vitro and in vivo the effect of combining the suppression of $p21^{Cip1}$ and $p27^{Kip1}$ in a Cdk4 R24C background, which makes this protein insensitive to the inhibition by INK4 proteins. We have confirmed that the inactivation of both $p21^{Cip1}$ and INK4 cooperates in suppressing cellular senescence *in vitro* and the development of tumors *in vivo*, especially those with mesenchimal origin. As the number of Cdk4 R24C, $p21^{Cip1}$ and $p27^{Kip1}$ mutant alleles increases, the latency of tumors shortens and their origin is restricted to endocrine cells. In collaboration with Clara Alvarez's group (Universidad de Santiago de Compostela) we have characterized a specific cell type present in this organ with features of stem cell. Mice homozygous for the three mutations (Cdk4 R24C, $p21^{Cip1}$ and $p27^{Kip1}$) died perinatally due to a systemic hypoplasia, contrary to the observations found in the intermediate genotypes which present almost complete penetrance of endocrine tumors. This hypoplasia is probably linked to replicative stress and defective cell cycle progression in the absence of all these cell cycle inhibitors.

Índice

Abreviaturas

ACTH: Hormona adrenocorticoptrópica *(**A**drenо**C**ortico**T**ropic **H**ormone)*

ATP: Adenosina-5'-Trifosfato *(**A**denosin-5'-**T**ri**P**hospate)*

DNA: Ácido Desoxirribonucleico *(**D**esoxiribo**n**ucleic **A**cid)*

cDNA: Ácido Desoxiribonucleico complementario *(**C**omplementary **D**esoxi ribo**n**ucleic **A**cid)*

RNA: Ácido **r**ibo**n**ucleico *(**R**ibo**n**ucleic **A**cid)*

mRNA: Ácido **R**ibo**n**ucleico **m**ensajero (**m**essenger **R***ibo**n**ucleic **A**cid)*

BrdU: Bromo**d**eoxi**u**ridina

BSA: ***B**ovine **S**erum **A**lbumin* (albúmina de suero bovino)

Células ES: Células madre embrionarias (***E**mbrionic **S**tem **C**ells*)

CKI: Inhididores de Cdks *(**C**dks **I**nhibitors)*

Cy3: indocarbocianina

CNIO: Centro **N**acional de **I**nvestigaciones **O**ncológicas

DCS: Suero de ternera (***D**onor **C**alf **S**erum)*

DMEM: Medio de Dubelcco modificado por Eagle (***D**ulbecco's **M**odified **E**agle's **M**edium)*

EDTA: *Ácido* etilendiaminotetraacético *(**E**thylene**d**iamine**t**etraacetic **A**cid)*

EdU: 5'-**E**tinilo-2'-**D**eoxi**U**ridina

FACS: Separación de células mediante fluorescencia *(**F**luorescence-**A**ctivated **C**ell **S**orter)*

FBS: ***F**etal **B**ovine **S**erum* (suero fetal bovino)

FSH: Hormona estimuladora del folículo *(**F**ollicle-**S**timulating **H**ormone)*

GH: Hormona del crecimiento *(**G**rowth **H**ormone)*

Ig: Inmuno**g**lobulina

IF: Inmuno**F**luorescencia

IHC: Inmunohistoquímica *(**I**mmuno**H**isto**C**hemistry)*

KDa: **K**ilo**Da**lton

KO: Animales o células derivadas de cepas deficientes en un gen (***k****nock-****o****ut*)

KI: Animales o células derivadas de cepas con mutación en un gen (***k****nock-****i****n)*

LH: Hormona luteinizante *(**L**uteinizing **H**ormone)*

MEFs: Fibroblastos embrionarios de ratón (***M****ouse* ***E****mbryonic* ***Fi****broblasts*)

mM: mili**M**olar

MACS: Magnetic **A**ctivated **C**ell **S**orting

MSH: Hormona estimuladora de los melanocitos *(**M**elanocyte-stimulating **H**ormone)*

nt: **n**ucleó**t**idos

pb: **p**ares de **b**ases

PBS: Tampón fosfato salino *(**P**hosphate **B**uffered **S**aline)*

PCR: Reacción en cadena de la polimerasa *(**P**olymerase **C**hain **R**eaction*)

PFA: **P**ara**F**orm**A**ldehído

PRL: **PR**o**L**actina

rpm: revoluciones **p**or **m**inuto

TSH: Tirotropina *(**T**hyroid-**S**timulating **H**ormone)*

SC: Célula madre *(**S**tem **C**ell)*

SDS: Dodecil sulfato sódico *(**S**odium **D**odecyl **S**ulphate)*

shRNA: Pequeñas horquillas de ARN *(**s**hort **h**airpin **RNA**)*

siRNA: Pequeño ARN de interferencia *(**s**hort **i**nterference **RNA**)*

UAM: Universidad **A**utónoma de **M**adrid

WB: Western **B**lot

WT: Animales o células derivadas de cepas silvestres (***W****ild* ***T****ype*

1.- Introducción

1.1.- Ciclo celular y cáncer

El ciclo celular es un conjunto de procesos que conducen al crecimiento celular y la división en dos células hijas. Estos procesos coordinados tienen la finalidad fundamental de conseguir la duplicación del DNA y la segregación de este material genético en dos células hijas mediante la división del citoplasma celular. El ciclo celular consta de cuatro fases. Las fases G1 (*gap 1*) y G2 (*gap 2*) son fases de crecimiento previas a las fases S y M respectivamente. La fase S o de Síntesis, es aquella en la que tiene lugar la replicación del DNA y la fase M o mitosis, es en la que se produce la segregación de los cromosomas en dos células hijas. La progresión a lo largo del ciclo celular está dirigida por varias proteínas con actividad quinasa. Entre estas quinasas se encuentran las quinasas dependientes de Ciclina (Cdks), las cuales son reguladores críticos en la transición de las diferentes fases del ciclo celular. En mamíferos, en un organismo adulto, la mayoría de las células se encuentran en un estado de quiescencia y por tanto se encuentran fuera de las fases de ciclo celular. Por analogía, esta fase de reposo se denomina G0. Bajo estímulos específicos estas células podrían volver a entrar en el ciclo celular (figura 1). Existe, un punto en la fase G1 denominado "punto de restricción" en el que las células continuarán el ciclo celular sin necesidad de estímulos externos (Pardee, 1974). En cuanto las células sobrepasan este punto, ya se han comprometido a entrar en la fase S donde tiene lugar la síntesis de DNA. La síntesis de DNA es un proceso que requiere gran cantidad de material y energía lo que hace lógico que una vez se han dado todas las condiciones para que esto suceda, se incite a la división celular con independencia de factores externos. Para asegurar una progresión correcta a lo largo del ciclo celular, las células han desarrollado una serie de puntos de control que las previenen de entrar en una fase del ciclo sin haber completado correctamente la fase anterior. Por ejemplo, las células se tienen que asegurar de haber alcanzado el tamaño homeostático adecuado, de que el DNA se duplica sin errores (punto de control del daño al DNA) o que la segregación cromosómica se produce de manera correcta (punto de control del correcto ensamblaje del huso mitótico).

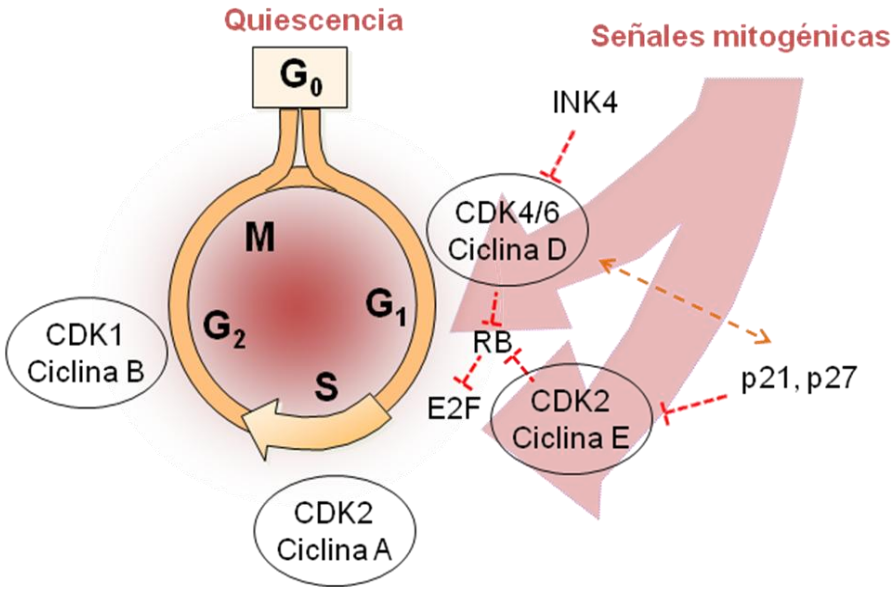

Figure 1.- **Representación esquemática del ciclo celular en mamíferos.** Se indican los complejos Cdk-Ciclina implicados en cada una de las fases así como los reguladores principales en la transición G1-S. El esquema supone una visión simplificada de la interacción entre Cdks, Ciclinas y una gran variedad de sustratos de estas moléculas (Modificado de (Malumbres and Barbacid, 2001))

El cáncer es un conjunto de enfermedades que se caracterizan por la proliferación incontrolada de células. Las células tumorales acumulan modificaciones tanto a nivel genético como epigenético que conllevan a la activación de señales mitogénicas constitutivas y a la falta de respuesta ante estímulos anti-proliferativos. Además, la mayoría de los tumores adquieren una inestabilidad genómica que conduce a mutaciones adicionales, así como una estabilidad cromosómica que produce modificaciones en el número de cromosomas. Todas estas alteraciones juntas implican, no solamente una ventaja proliferativa, sino además un incremento en la susceptibilidad a la acumulación de nuevas alteraciones genéticas que contribuyen a la progresión tumoral y a la adquisición de fenotipos más agresivos. Todavía no esta clara la relación que existe entre los diferentes cánceres y sus subtipos, ni siquiera cuáles son las rutas moleculares alteradas para generar una célula tumoral o si estas rutas son las mismas en todos los tipos tumorales. De hecho, las alteraciones fisiológicas más comunes que deben ocurrir en la

célula para dar lugar a una célula tumoral están en continua revisión. Actualmente se han definido hasta 10 características de las células tumorales: la evasión de la muerte celular y la senescencia, un potencial replicativo ilimitado, una señalización celular constitutivamente activada, la capacidad invasiva y de metástasis, la capacidad para mantener estimulada la angiogénesis, la capacidad evasiva a los puntos de control del daño al DNA y al estrés replicativo, altos niveles de estrés metabólico y mitótico, inestabilidad genómica y evasión de la vigilancia del sistema inmune (Negrini et al., 2010).

1.2.- Progresión a lo largo de las fases G1 y S

1.2.1.- Los complejos Cdks-Ciclinas como directores de la progresión a lo largo de las fases G1 y S

La fase S del ciclo celular es aquella en donde tiene lugar la replicación del DNA y la fase G1 es aquella fase de crecimiento y transición que precede a la fase S. Las Cdks son proteínas quinasas heterodiméricas compuestas por una subunidad catalítica (Cdk) y una subunidad reguladora (Ciclina). El número de Cdks que existen en la naturaleza ha ido incrementándose con la evolución. Así, mientras organismos unicelulares como las levaduras *Saccharomices Cerevisiae* o *Schizosaccharomyces pombe* solo necesitan una Cdk que se une a diferentes Ciclinas dependiendo de la fase del ciclo, organismos más evolucionados han incrementado el número que poseen de estás proteínas. De este modo, basándonos en la homología en la secuencia, el genoma humano contiene 25 loci que codifican para proteínas Ciclina y 21 genes que codifican para Cdks, aparte de 5 genes adicionales con una homología más lejana que codifican para quinasas similares-a-Cdk (*Cdk-like*) (Malumbres and Barbacid, 2009; Malumbres et al., 2009). Sin embargo, sólo Cdk1, Cdk2, Cdk4 y Cdk6 han sido implicadas en la progresión en el ciclo celular. Sin embargo, estudios mediante ratones modificados genéticamente han demostrado a la Cdk1 como la única esencial para la progresión en el ciclo celular (Santamaria et al., 2007), mientras que las Cdks de interfase son sólo esenciales para la proliferación en células

especializadas. Esto no quiere decir que las Cdks de interfase no intervengan en los ciclos celulares normales en esas células en las que son indispensables. La expresión de las subunidades catalíticas o Cdks es constante a lo largo del ciclo celular y su actividad se ve regulada por su unión a diferentes Ciclinas durante las diferentes fases de ciclo. El esquema clásico sugiere que durante la fase G1, las señales mitogénicas conllevan la expresión de las proteínas Ciclinas de tipo D (D1, D2 y D3) las cuales se unen y activan a Cdk4 y Cdk6. Esto produce la activación del complejo lo que resulta principalmente en la fosforilación de las proteínas de la familia pRB. La fosforilación de pRB es parcial y permite la expresión de las Ciclinas de fase S (Ciclinas E), las cuales se unen a Cdk2 produciendo la hiperfosforilación de la pRB. La función de Cdk2, sin embargo, está en entredicho dado que esta proteína es prescindible en mitosis (Malumbres and Barbacid, 2009). La inactivación de pRB provoca la liberación de los factores de transcripción formados por los diferentes complejos de las familias E2F-DP lo que da lugar a la expresión de los genes requeridos para las fases S y M (figura 2).

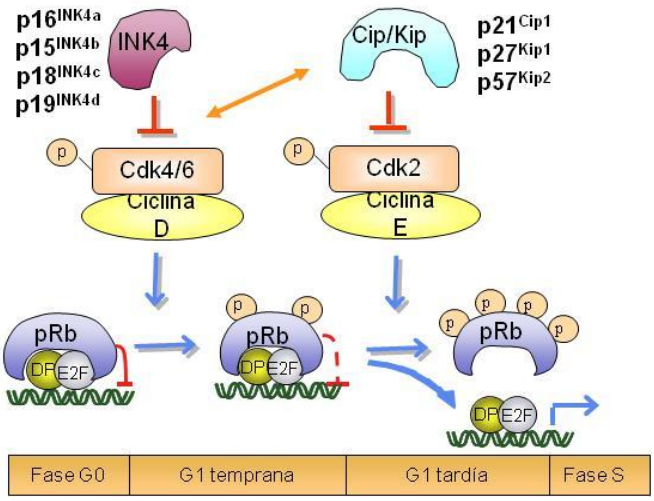

Figura 2.- Progresión de G0 a la fase S en células de mamífero. La fosforilación inicial de la proteína pRB por los complejos Cdk4/6-Cicilna D permite la transcripción de algunos genes. La fosforilación posterior por complejos Cdk2-Ciclina E puede ayudar a completar la hiperfosforilación de pRB lo que permite la liberación total de los complejos E2F-DP y la transcripción de todas sus proteínas diana. Los inhibidores de la familia Cip/Kip regulan la actividad de los complejos que forman las Ciclinas tipo D y E, mientras que los inhibidores de la familia INK4 se unen exclusivamente a Cdk4 y Cdk6.

1.2.2.- Inhibidores de las Cdks de las fases G1-S

Aparte de por la fluctuación en la expresión de las subunidades reguladoras (Ciclinas), la actividad de las Cdks se encuentra regulada por inhibidores específicos (CKIs). Estas proteínas contrarrestan la actividad de los complejos Cdks mediante el bloqueo directo de su activación o el impedimento del acceso al sustrato o al ATP. Existen dos familias conocidas de CKIs, la familia INK4 compuesta por los miembros $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ y $p19^{INK4d}$ y la familia Cip/Kip compuesta por las proteínas $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ (figura 2). Las proteínas de la familia INK4 (*inhibitors of Cdk4)* inhiben la progresión entre las fases G1/S mediante la inhibición directa por unión a Cdk4 y Cdk6. Estas proteínas se caracterizan por la posesión de un dominio estructural común, las repeticiones de ankirinas. Las repeticiones de ankirina consisten en pares de hélices α unidas por los lados y conectadas entre ellas por motivos horquilla. A través de estos dominios estructurales se produce la unión con la parte la parte catalítica de las proteínas Cdk4 y Cdk6, interaccionando tanto con aminoácidos de la parte amino como de la carboxilo terminal. Esta unión no es solapante con el dominio de unión a las proteínas Ciclinas, sin embargo induce la inhibición de los complejos Cdk-Ciclina a través de dos sucesos, provoca un cambio alostérico en el sitio de unión de las Ciclinas que reduce la afinidad a la unión y reduce de manera significativa la unión al ATP (figura 3) (Pavletich, 1999).

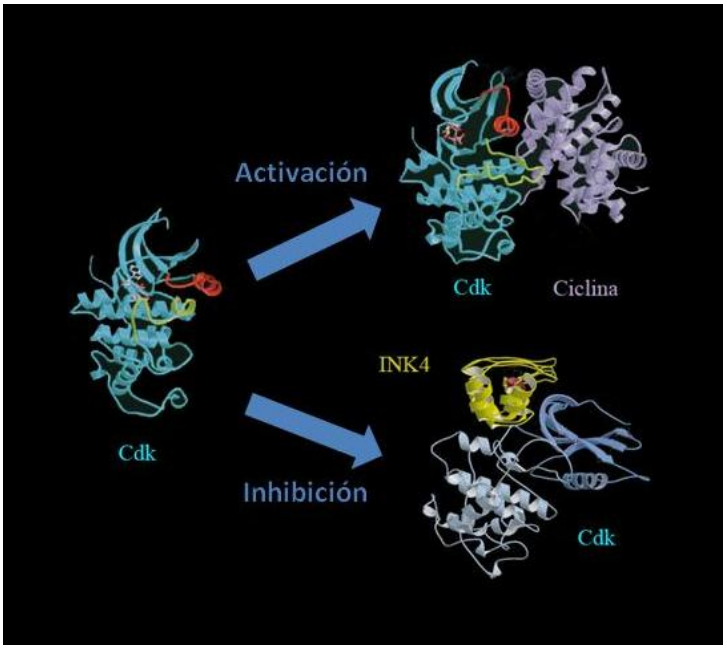

Figura 3.- Modelo de la estructura tridimensional Cdk-Ciclina-INK4. Esquema de la estructura tridimensional de las Cdk y su interacción con sus reguladores las proteínas Ciclina (activación) o la inhibición por proteínas de la familia INK4. Adaptado de (Pavletich, 1999) y (Brotherton et al., 1998).

Los miembros de la familia Cip/Kip tienen, sin embargo, papeles diferentes dependiendo del complejo Cdk-Ciclina con el que se asocien. La asociación a complejos formados por las proteínas Cdk2 y Cdk1 provocaría el bloqueo de la actividad quinasa, mientras que el papel de la unión con complejos Cdk4/6-Ciclina D permanece confuso. Ha habido estudios que decían que esta unión era necesaria para la activación de los complejos Cdk4/6-Ciclina D (Cheng et al., 1999; Lam et al., 2000; Sicinski et al., 1996), mientras que otro afirmaba que este suceso no era necesario (Bagui et al., 2003). Incluso, se ha llegado a postular que la unión a complejos Cdk4 y Cdk6 se produce para permitir la liberación y activación de los complejos Cdk2-Ciclina E (Sherr and Roberts, 1999). Últimamente, se están caracterizando otros papeles para las proteínas de esta familia que son incluso más destacables a raíz del estudio realizado en modelos animales modificados genéticamente en donde Cdk2 se descarta como diana principal de las proteínas $p21^{Cip1}$ y $p27^{Kip1}$ en la inhibición del ciclo celular y la supresión tumoral (Martin et al., 2005). Por ejemplo, $p21^{Cip1}$ se ha descrito como regulador de la transcripción independiente de la regulación de Cdk2. $p21^{Cip1}$ también es un regulador de la apoptosis en la vía dependiente de p53 y está implicado en procesos de reparación del DNA mediante la inhibición de la proteína PCNA necesaria para la progresión de la fase S, permitiendo de este modo la reparación del DNA. $p21^{Cip1}$ se ha relacionado también con el mantenimiento de la capacidad pluripotente de las células madre en varios tejidos (Abbas and Dutta, 2009). Del mismo modo, otros papeles de $p27^{Kip1}$ diferentes del ya mencionado como regulador del ciclo celular y su relación con los complejos Cdk-Ciclina han sido descritos. Cuando se encuentra en el citoplasma, $p27^{Kip1}$ regula la motilidad celular mediante la interacción con RhoA (Besson et al., 2004). Además, igual que $p21^{Cip1}$, $p27^{Kip1}$ también ha sido relacionado con el mantenimiento de la capacidad pluripotente de las células madre y su autorregeneración en diversos tejidos (Li et al., 2009; Pechnick et al., 2008).

El balance correcto entre actividad Ciclina-Cdk y su inhibición por parte de los CKIs es fundamental para una correcta progresión a lo largo del ciclo celular. La desregulación de los complejos Cdk-Ciclina aparece como uno de los procesos clave en numerosos tipos tumorales. Gran número de estas mutaciones se encuentran bien caracterizadas y afectan

tanto a los complejos Cdk-Ciclina directamente como a sus diferentes reguladores directos (Malumbres and Barbacid, 2001). Así, Cdk4 aparece alterada en un pequeño número de pacientes de melanoma presentando una mutación puntual que modifica la arginina 24 a cisteína e impide la unión de los inhibidores de la familia INK4 a esta proteína (Wolfel et al., 1995; Zuo et al., 1996). Cdk4 y Cdk6 son sobreexpresadas o amplificados en diferentes tumores incluyendo sarcomas, gliomas, tumores de mama, linfomas y melanomas. La desregulación de las Ciclinas de tipo D y los inhibidores de la familia INK4 es característica común a la mayoría de los tipos tumorales. Esta desregulación conllevaría a la hiperactividad de las quinasas Cdk4 y Cdk6. Aunque los inhibidores de la familia INK4 son ejemplos claros de proteínas que actúan como supresores tumorales, el papel de los inhibidores de la familia Cip/Kip en procesos tumorales es algo más controvertido y se ha visto que tanto $p21^{Cip1}$ como $p27^{Kip1}$ pueden actuar como supresores tumorales y como oncogenes dependiendo del contexto. Para llegar a este término ha sido de gran utilidad el uso de modelos animales modificados genéticamente y el análisis como marcadores de prognosis de diversos tipos tumorales humanos. Así, por ejemplo, la mutación puntual C94T se ha encontrado en tumores de mama y hace a $p21^{Cip1}$ incapaz de inhibir a la actividad Cdk (Balbin et al., 1996). En este mismo tipo de tumores, altos niveles citoplasmáticos de $p21^{Cip1}$ fueron asociados con altos niveles de p53 y Ciclina B y correlacionaban con pobre prognosis (Abbas and Dutta, 2009). En el caso de $p27^{Kip1}$ cabe destacar que sus niveles de expresión aparecen disminuidos en gran número de cánceres. Clínicamente, se utiliza los niveles de expresión de p27 como marcador de prognosis, asociándose mal prognosis a bajos niveles de $p27^{Kip1}$. Por otro lado, existe un estudio realizado a partir de un modelo de ratón modificado genéticamente en donde se sustituye el $p27^{Kip1}$ endógeno por un $p27^{Kip1}$ mutado, incapaz de unirse a las Ciclinas, y el cual produce tumores de manera más agresiva que los animales sin esta proteína (Besson et al., 2007). Estos datos en conjunto, incitan a pensar en un doble papel tanto de $p21^{Cip1}$ como de $p27^{Kip1}$ en tumorigenésis, pudiendo actuar tanto como genes supresores de tumores como oncogenes. En el caso de $p27^{Kip1}$, esto podría ser explicado a través de la existencia de una relación de $p27^{Kip1}$ con la ruta de Rho implicada en motilidad celular,

migración celular, invasión y metástasis y podría ser una respuesta a por qué raramente esta proteína es eliminada completamente en tumores humanos (Chu et al., 2008).

1.3.- Modelos animales como método de estudio

El uso de modelos animales modificados genéticamente para el estudio de todo tipo de proteínas y su implicación con cáncer ha sido muy utilizado a lo largo de toda la historia. Gran número de modelos animales han sido desarrollados para analizar el rol de los complejos Cdk-Ciclina y su interacción con sus diferentes inhibidores (tabla 1). Uno de los primeros modelos descritos de una proteína implicada en ciclo celular fue el *knock-out* de pRB (Jacks et al., 1992; Lee et al., 1992). Los animales que carecen de esta proteína mueren en estadios embrionarios debido a defectos en eritropoyesis y con alta presencia de muerte neuronal. Los Animales con solo una copia de este gen desarrollan tumores en la hipófisis con largas latencias.

Mediante el uso de modelos animales se ha podido comprobar que, en mamíferos, la única Cdk imprescindible para la progresión en el ciclo celular es la Cdk mitótica o Cdk1 y que la eliminación de esta proteína del genoma provoca la letalidad embrionaria en ratones en estadio de dos células debido a la imposibilidad de completar el ciclo celular (Santamaria et al., 2007). La eliminación individual de cualquiera de las Cdks de interfase, Cdk2, Cdk4 o Cdk6, no provoca la letalidad embrionaria, aunque sí resulta en severos defectos en tejidos especializados. Así, animales sin Cdk4 presentan diabetes debido a defectos en la proliferación de células β del páncreas y de las células lactotrofas de la hipófisis (Rane et al., 1999). Los ratones sin Cdk2 son estériles debido a problemas en la realización de la mitosis (Ortega et al., 2003) y animales *knock-out* para Cdk6 presentan anemia y defectos en la proliferación de determinadas células hematopoyéticas destacando la línea eritroide. Este fenotipo se vuelve más agresivo cuando se combina la deficiencia de Cdk6 con la falta de Cdk4 y estos animales mueren en estadio embrionario con anemia severa por la falta de eritrocitos (Malumbres et al., 2004). La eliminación

conjunta de todas las Cdks de interfase (Cdk2, Cdk4 y Cdk6), agudiza estos defectos hematopoyéticos, pudiendo llegar los embriones a la mitad de la gestación (Santamaria et al., 2007).

La eliminación de las Ciclinas en modelos animales modificados genéticamente también se ha utilizado para investigar el papel de estos complejos en el ciclo celular y su implicación en cáncer. La eliminación de las Ciclinas D provoca, de la misma manera que la eliminación de las respectivas Cdks, problemas en tejidos específicos. Así, la eliminación de la Ciclina D1 provoca principalmente defectos en la retina y en la mama durante el embarazo (Fantl et al., 1995; Sicinski et al., 1995). Los ratones sin Ciclina D2 presentan esterilidad en las hembras, problemas en el desarrollo del cerebelo y una proliferación reducida de células B (Huard et al., 1999; Lam et al., 2000; Sicinski et al., 1996). Animales deficientes en Ciclina D3 presentan un timo subdesarrollado con falta de maduración de linfocitos T (Sicinska et al., 2003). La deficiencia combinada de dos de estas Ciclinas que se asocian a Cdk4 y Cdk6 presenta los mismos fenotipos que los animales simples más agresivos, resultando en la muerte o bien en estadíos embrionarios o de manera perinatal (Ciemerych et al., 2002). La ausencia total de estas Ciclinas de tipo D implica la aceleración de la muerte en estado embrionario y la suma de todos los fenotipos presente en los modelos simples (Kozar et al., 2004).

Se han generado diversos modelos animales de las CKIs para analizar el papel de estas proteínas en el ciclo celular, su relación con los complejos Cdk-Ciclina y su posible papel en cáncer. Con este fin, se han generado modelos *knock-out* de todas las proteínas de la familia INK4 y de la familia Cip/Kip. Salvo los animales *knock-out* para $p19^{INK4d}$, el resto de modelos deficientes en una de las proteínas de la familia INK4 presenta un fenotipo tumoral, aunque este se produce en latencias muy largas. De este modo, animales deficientes para $p16^{INK4a}$ presentan principalmente linfomas y sarcomas (Sharpless et al., 2001). Los animales sin $p15^{INK4b}$ presentan angiosarcomas (Latres et al., 2000). Los animales *knock-out* para $p18^{INK4c}$ presentan un aumento en el tamaño corporal y en el de algunos órganos y tumores de hipófisis (Franklin et al., 1998; Latres et al., 2000). La falta de $p19^{INK4d}$, por el contrario, no presenta mayor fenotipo que la atrofia de los testículos en

machos, aunque estos siguen siendo fértiles (Zindy et al., 2000). Los estudios de combinaciones dobles de inhibidores de esta familia no resultó un diferencias significativas con respecto a las eliminaciones simples (Latres et al., 2000; Ramsey et al., 2007). Sin embargo, cabe destacar el estudio del doble KO para $p16^{INK4a}$ y $p15^{INK4b}$. Estas proteínas se encuentran en el mismo locus genómico por lo que ha sido bastante complicada su eliminación simultánea (Krimpenfort et al., 2007). Este modelo presenta un mayor espectro y una menor latencia en los tumores aparecidos lo que sugiere una capacidad compensatoria por parte de una de las proteínas de la familia cuando la otra no se haya presente. Un modelo animal desarrollado muy interesante en el estudio del papel de los inhibidores INK4 es el modelo *knock-in* desarrollado en la proteína Cdk4 que sustituye a la Cdk4 endógena por una que sustituye la arginina 24 por una cisteína. Esta mutación es homóloga a la que ya hemos mencionado en humanos y que fue descubierta en un análisis masivo en melanomas humanos (Wolfel et al., 1995). La sustitución R24C previene la unión de los inhibidores de la familia Ink4 a la proteína Cdk4. La introducción de la esta mutación en el genoma de ratón resulta en la aparición de diversos tumores con una penetrancia completa (Sotillo et al., 2001).

En el caso de la eliminación de proteínas de la familia Cip/Kip, animales sin $p21^{Cip1}$ presentan fundamentalmente sarcomas histiocíticos con muy larga latencia (Martin-Caballero et al., 2001). La falta de $p27^{Kip1}$ en ratones resulta en un incremento del tamaño corporal y de los órganos, esterilidad en hembras y tumores de pituitaria con alta penetrancia, pero con larga latencia (Kiyokawa et al., 1996; Nakayama et al., 1996). Como ya he comentado, existe un modelo que sustituye el $p27^{Kip1}$ endógeno por un $p27^{Kip1}$ mutado incapaz de unirse a las Ciclinas que produce tumores de manera más agresiva que los animales carentes de esta proteína (Besson et al., 2007). Cuando se elimina $p57^{Kip2}$ en ratones, los animales mueren perinatalmente debido a defectos en el desarrollo (Yan et al., 1997; Zhang et al., 1997). Los fenotipos de los animales dobles mutantes para los inhibidores Cip/Kip o bien no han sido descritos, como el caso de $p21^{Cip1}$ y $p27^{Kip1}$, o presentan cooperación en términos de desarrollo (Zhang et al., 1998; Zhang et al., 1999).

También se han desarrollado modelos animales combinando la depleción de inhibidores Cip/Kip con inhibidores de la familia INK4. En este caso, sí se ha visto cooperación en la latencia e incidencia de los tumores. En el caso de ratones sin $p18^{INK4c}$ y $p21^{Cip1}$, estos disminuyeron ligeramente la latencia en tumores de hipófisis, aumentando su incidencia (Franklin et al., 2000). Animales sin $p27^{Kip1}$ y $p18^{INK4c}$ cooperaron de manera más agresiva que los mencionados anteriormente en la producción de tumores de hipófisis (Franklin et al., 1998). También el módelo *knock-in* de Cdk4 R24C ha sido combinado con la eliminación de $p27^{Kip1}$. Estos animales presentan una cooperación dramática en la formación de tumores, apareciendo tumores de hipófisis con una penetrancia completa y una vida media de aparición del tumor menor de tres meses (Sotillo et al., 2005). Los inhibidores Cip/Kip cooperan con pRB en la protección contra tumores hipofisarios, como podemos comprobar en modelos animales combinados para estas proteínas (Brugarolas et al., 1998; Park et al., 1999)

Tabla 1. **Modelos de tumores en ratones genéticamente modificados en reguladores de G1/S**[a]

Cepa[b]	Fenotipo	Referencias
Modelos de Cdks		
Cdk1	Letalidad embrionaria en las primeras divisiones. Incapacidad para completar el ciclo celular	(Santamaria et al., 2007)
Cdk2	Esterilidad por defectos en la meiosis	(Ortega et al., 2003)
Cdk4	Diabetes y problemas en la proliferación de células tipo β páncreas y células lactotrofas de la hipófisis	(Rane et al., 1999)(Tsutsui et al., 1999)
Cdk6	Anemia y defectos en la proliferación de células hematopoiéticas	(Malumbres et al., 2004)
$Cdk4^{R24C}$ (No se une a INK4)	Diversos tipos de tumores con penetrancia completa	(Rane et al., 1999; Sotillo et al., 2001)
Modelos de Ciclinas		
Ciclina D1	Defectos en la retina y en el tejido mamario durante el embarazo	(Fantl et al., 1995)
Ciclina D2	Esterilidad en las hembras. Problemas en el desarrollo del cerebelo y proliferación reducida de células B	(Lam et al., 2000; Sicinski et al., 1996)
Ciclina D3	Problemas en la maduración de linfocitos T	(Sicinska et al., 2003)

Tabla 1. **Modelos de tumores en ratones genéticamente modificados en reguladores de G1/S**[a] **(Continuación)**

Cepa[b]	Fenotipo	Referencias
Modelos de inhibidores		
$p16^{INK4a}$	Linfomas de células B y sarcomas con un 25% de incidencia.	(Sharpless et al., 2001)
$p15^{INK4b}$	Desórdenes linfoproliferativos y hemangiosarcomas con una incidencia menor del 10%.	(Latres et al., 2000)
$p18^{INK4c}$	Alta incidencia de tumores de hipófisis. Otras neoplasias que incluyen tumores de testículo y feocromocitomas.	(Latres et al., 2000)
$p19^{INK4d}$	Normales salvo que, aunque son fértiles, los machos presentan atrofia testicular	(Zindy et al., 2000)
$pRb^{+/-}$	Tumores de origen endocrino: hipófisis, tiroides y médula adrenal. La mutación en homicigosidad es letal embrionaria.	(Jacks et al., 1992; Lee et al., 1992)
$p21^{Cip1}$	Los ratones desarrollan sarcomas, linfomas de células B y tumores epiteliales (pulmón, piel, células de Leydig) a los 16 meses de edad.	(Martin-Caballero et al., 2001)
$p27^{Kip1}$	Aumento de tamaño. Esterilidad en las hembras. Displasia de la retina y alta penetrancia de tumores de hipófisis. Haploinsuficiente en la supresión tumoral.	(Kiyokawa et al., 1996; Nakayama et al., 1996)
$p27^{CK-}$ (no se une a Ciclinas)	Mismos fenotipos del *knock-out* aunque más agresivos. Hiperplasia en varios tejidos y disminución de la latencia en tumores de hipófisis	(Besson et al., 2007)
$p18^{INK4c}$;$p21^{Cip1}$	Hiperplasia neuroendocrina gástrica y tumores de pulmón bronqueoalveolares.	(Franklin et al., 2000)
$p18^{INK4c}$;$p27^{Kip1}$	Incremento del tamaño de los órganos, algunos hiperplásicos y disminución de la latencia en tumores de hipófisis	(Franklin et al., 1998)
$Cdk4^{R24C}$;$p27^{Kip1}$	Penetrancia completa en el desarrollo de tumores; destacan los de hipófisis muy agresivos y con cortas latencias	(Martin-Caballero et al., 2004)
$pRb^{+/-}$;$p21^{Cip1}$	Menor latencia en adenocarcinomas de hipófisis.	(Brugarolas et al., 1998)
$pRb^{+/-;}p27^{Kip1}$	Tumores de hipófisis tempranos y más agresivos.	(Park et al., 1999)

[a] No están representadas aquellas cepas de ratones dobles o triples mutantes que no presentan diferencias signficativas con los ratones mutantes sencillos (*e.g*. dobles mutantes $p15^{INK4b}$;$p18^{INK4c}$).

[b] Mutaciones en homocigosis a no ser que se especifique otra cosa.

1.4.- La hipófisis

La hipófisis es un órgano central endocrino que regula funciones fisiológicas básicas como pueden ser el crecimiento, la reproducción o la homeostasis metabólica. En mamíferos, la hipófisis se divide en tres lóbulos: la hipófisis posterior (PP: *Posterior Part*), el lóbulo intermedio (IL: *Intermediate Layer*) (atrófico en humanos) y la hipófisis anterior (AP: *Anterior Part*) (figura 3). Entre la hipófisis anterior y el lóbulo intermedio existe una hendidura en donde se encuentra un tipo celular específico sin capacidades endocrinas. Esta capa de células individuales se ha denominado como zona marginal. Las diferentes funciones fisiológicas que dirige la hipófisis son llevadas a cabo por seis tipos celulares especializados que secretan hormonas específicas al torrente sanguíneo. Estos tipos celulares especializados se localizan en la hipófisis anterior y el lóbulo intermedio y son definidos precisamente por la hormona específica que secretan. Son seis: corticotropas que secretan hormona adrenocorticotrópica (ACTH), tirotropas que generan tirotropina (TSH), somatotropas que producen hormona del crecimiento (GH), lactotropas que generan prolactina (PRL), gonadotropas que secretan hormona luteinizante (LH) y hormona estimuladora del folículo (FSH) y melanotropas que producen hormona estimuladora de los melanocitos (MSH). Todas estas células especializadas se encuentran en la hipófisis anterior salvo las melanotropas que se localizan en el lóbulo intermedio. La hipófisis adulta se origina desde el primordio embrionario neuroectodérmico conocido como bolsa de Rathke (figura 4). La formación de la hipófisis esta controlada de manera espacio-temporal durante el desarrollo a través de gradientes

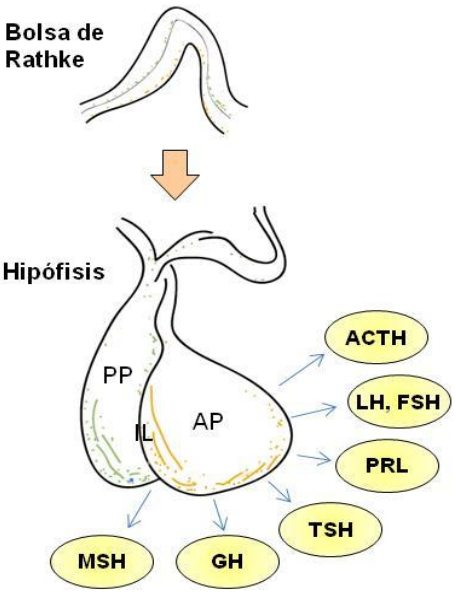

Figura 4.- Esquema general de la glándula hipofisaria desde su generación desde la bolsa de Rathke hasta su estado en adulto. Se indican las hormonas que se secretan. PP: Hipófisis posterior; IL: lóbulo intermedio; AP: hipófisis anterior

de señalización llevados a cabo por diversos factores entre los que destacan las vías de señalización de Notch y Wnt (Zhu et al., 2007). La regulación de de la capacidad proliferativa de las células de la pituitaria en el organismo adulto no ha sido completamente establecida, aunque diferentes poblaciones celulares han sido postuladas como las células madre del tejido (Chen et al., 2005; Vankelecom, 2007).

1.4.1.- Reguladores del ciclo celular implicados en tumorigénesis en la hipófisis.

La aparición de adenomas en la hipófisis es un suceso bastante común en humanos. Datos de análisis de autopsias regulares sugieren que entre el 17 y 25% de la población desarrolla adenomas de hipófisis. De todos los tumores hipofisarios, aproximadamente entre el 3,5 y el 8% son diagnosticados antes de los 20 años (Keil and Stratakis, 2008). Además, unos dos tercios de los tumores de hipófisis expresan y secretan diversas hormonas lo que provoca la asociación a diversos síndromes de carácter endocrino. Diversas alteraciones genéticas y epigéneticas de reguladores de ciclo celular han sido encontradas en tumores de la hipófisis en humanos. Como ejemplo valgan las mutaciones encontradas en la proteína H-RAS en los codones 12 o 18 (Pei et al., 2004) o la pérdida de heterozigosidad de pRB en el 100% de tumores malignos o muy invasivos (Pei et al., 1995). Esta proteína es una de las que se ve más frecuentemente inactivada en tumores hipofisiarios mediante hipermetilación de su promotor. Los niveles de expresión de las proteínas de la familia INK4 también son frecuentemente reprimidos por este método habiéndose encontrado la hipermetilación del promotor de p16 en valores que oscilan entre el 40 y el 70% dependiendo del tumor hipofisario analizado (Machiavelli et al., 2008; Ogino et al., 2005). Los niveles de expresión de los inhibidores de la familia Cip/Kip también se han encontrado reducidos en diversos tipos de tumores de la hipófisis (Bamberger et al., 1999; Neto et al., 2005). Por el contrario, se ha encontrado un aumento en los niveles de expresión de las proteínas Ciclinas. De este modo, los niveles de Ciclina D1 se hayan aumentados entre un 30 y 50% en somatotropinomas y tumores que no secretan hormonas (Simpson et al., 2001) y los niveles de Ciclina D3 se ven incrementados en un 68% de los adenomas de hipófisis (Saeger et al., 2001). Estos datos correlacionan con los datos observados en modelos animales y descritos anteriormente en donde la

ausencia de proteínas implicadas en ciclo celular como pRB, $p27^{Kip1}$ o $p18^{INK4c}$ da lugar al desarrollo de tumores.

Para analizar el efecto en la transformación celular y la tumorigénesis de los inhibidores CKI, tanto la familia INK4 como los de la familia Cip/Kip, hemos trabajado en esta tesis con diferentes modelos animales. Por un lado el modelo de Cdk4 R24C que le hace insensible a la inhibición por proteínas de la familia INK4 (Rane et al., 1999). Este modelo lo hemos cruzado con animales deficientes para $p21^{Cip1}$ (Brugarolas et al., 1995) y posteriormente con animales deficientes para $p27^{Kip1}$ (Nakayama et al., 1996). Este modelo triple mutante constituye un modelo ideal para analizar el efecto a nivel proliferativo y de formación tumoral en ausencia de inhibidores de ciclo celular. Del mismo modo, la hipófisis se convierte en un órgano central de estudio debido a la alta penetrancia que tienen este tipo de tumores y la variedad que encontramos en el origen de este tipo de neoplasias en los diferentes modelos animales. Este hecho nos hace plantearnos la desregulación de las células madre de este órgano como punto de partida en la tumorigénesis hipofisaria lo que nos plantea la caracterización de este tipo celular en la hipófisis. Esta memoria se centra por lo tanto en el análisis de estos modelos animales en los que vamos introduciendo progresivamente las diferentes mutaciones y la caracterización tanto a nivel celular como in vivo de los fenotipos que presentan.

2.- Objetivos

El objetivo principal de esta tesis es analizar el efecto conjunto producido por la ausencia de los inhibidores de ciclo celular de las familias INK4 y Cip/Kip en la regulación del ciclo celular y la tumorigénesis. Para ello nos planteamos los siguientes objetivos:

1.-Analizar el efecto combinatorio de la ausencia de inhibición de los inhibidores INK4 en la proteína Cdk4 mediante el uso del modelo Cdk4 R24C y la falta de la proteína $p21^{Cip1}$ tanto *in vivo* como *in vitro*.

2.- Caracterizar la ausencia combinada de los inhibidores Cip/Kip, $p21^{Cip1}$ y $p27^{Kip1}$, en el fondo genético Cdk4 R24C.

3.- Usando la hipófisis como modelo de tumorigénesis, caracterizar el efecto de la desregulación de los diferentes inhibidores de ciclo celular Cip/Kip o INK4 (mediante el uso del modelo animal Cdk4 R24C) en posibles células progenitoras y en el desarrollo tumoral.

3.- Materiales, métodos y resultados

Manuscritos prensentados:

3.1.- Cooperación genética entre los inhibidores INK4 y p21^{Cip1} en senescencia celular y supresión tumoral

Quereda, V., J. Martinalbo, P. Dubus, A. Carnero, and M. Malumbres. 2007. Genetic cooperation between p21^{Cip1} and INK4 inhibitors in cellular senescence and tumor suppression. Oncogene. 26:7665-74.

3.2.- Regulación del ciclo celular en el desarrollo de la hipófisis y sus enfermedades

Quereda, V., and M. Malumbres. 2009. Cell cycle control of pituitary development and disease. J Mol Endocrinol. 42:75-86.

3.3.- Un nicho de células GFRa2/Prop1/Stem (GPS) en la hipófisis

Garcia-Lavandeira, M., V. Quereda, I. Flores, C. Saez, E. Diaz-Rodriguez, M.A. Japon, A.K. Ryan, M.A. Blasco, C. Dieguez, M. Malumbres, and C.V. Alvarez. 2009. A GRFa2/Prop1/stem (GPS) cell niche in the pituitary. PLoS One. 4:e4815.

3.4.- Las familias de inhibidores INK4 y Cip/Kip previenen el estrés replicativo durante el desarrollo

Quereda V, Dubus P, Cañamero M, Temple S and Malumbres M. 2010. Ink4 and Cip/Kip family inhibitors prevent replicative stress during development. (Manuscript in preparation)

3.1.- Cooperación genética entre los inhibidores INK4 y $p21^{Cip1}$ en senescencia celular y supresión tumoral

Las familias de inhibidores de ciclo celular Cip/Kip e INK4 están involucradas en senescencia celular y supresión tumoral. Algunas de estas proteínas como $p21^{Cip1}$, $p16^{INK4a}$ y $p15^{INK4b}$ son cooexpresadas en respuestas a señales antiproliferativas como la senescencia celular, lo que provoca el arresto del ciclo celular. Para comprender el papel de estos inhibidores y un posible efecto sinérgico, hemos caracterizado las propiedades de crecimiento y el comportamiento en la senescencia de células primarias deficientes en $p21^{Cip1}$ que expresan de manera endógena el mutante Cdk4 R24C [alelo Cdk4 (R)] que hace a esta proteína insensible a la inhibición por proteínas de la familia INK4. La inactivación conjunta de de ambas rutas, $p21^{Cip1}$ e INK4, coopera dramáticamente en suprimir la senescencia celular *in vitro*. Estos dobles mutantes se comportan como células inmortales y muestran una elevada sensibilidad a la transformación por oncogenes. Además, animales dobles mutantes para las rutas INK4 y $p21^{Cip1}$ presentan una elevada incidencia de sarcomas, lo que sugiere que existe una cooperación significativa entre estas dos familias de inhibidores de ciclo celular en respuestas a la senescencia y a la supresión tumoral *in vivo*

Quereda, V., J. Martinalbo, P. Dubus, A. Carnero, and M. Malumbres. 2007. Genetic cooperation between $p21^{Cip1}$ and INK4 inhibitors in cellular senescence and tumor suppression. Oncogene. 26:7665-74.

Contribución del doctorando al trabajo

Victor Quereda realizó los experimentos con células, colaborando en la generación de la mayoría de los materiales necesarios para su desarrollo (obtención de vectores, MEFs,…). Se encargó del mantenimiento de la colonia de animales, organizando los cruces y realizando las necropsias a los animales que aparecen en las diferentes gráficas. Colaboró en el diseño de los experimentos y en la escritura del artículo.

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ORIGINAL ARTICLE

Genetic cooperation between p21^{Cip1} and INK4 inhibitors in cellular senescence and tumor suppression

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Cell-cycle inhibitors of the Cip/Kip and INK4 families are involved in cellular senescence and tumor suppression. Some of these proteins, p21^{Cip1}, p16^{INK4a} and p15^{INK4b}, are coexpressed in response to antiproliferative signals such as cellular senescence resulting in cell-cycle arrest. To understand the roles of these inhibitors and their synergistic effect, we have characterized the growth properties and senescent behavior of primary cells deficient in p21^{Cip1} and expressing an endogenous Cdk4^{R24C} (cyclin-dependent kinase) mutant (Cdk4^{R24C} knock-in cells) insensitive to INK4 proteins. Inactivation of both p21^{Cip1} and INK4 pathways strongly cooperate in suppressing cellular senescence *in vitro*. These double mutant cells behavior as immortal cultures and display high sensitivity to cellular transformation by oncogenes. Moreover, mice double mutant in the INK4 and p21^{Cip1} pathways (Cdk4^{R24C}; p21^{Cip1}-*null* mice) display an increased incidence of specific sarcomas, suggesting a significant cooperation between these two families of cell-cycle inhibitors in senescence responses and tumor suppression *in vivo*.
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Keywords: cell-cycle inhibition; cyclin-dependent kinase; senescence; tumor development; p21^{Cip1} and INK4 inhibitors

Introduction

The involvement of cell-cycle regulators in human cancer has been extensively established in the past years (Sherr, 2000; Malumbres and Carnero, 2003). Most alterations in the cell-cycle target regulators of the G_1/S transition, a period where cells decide whether to enter into the cell cycle upon mitogenic stimuli, or staying quiescent in response to antimitogenic or senescence signals. The retinoblastoma protein (pRb) pathway seems to play a key role in the regulation of these cellular processes since pRb and their regulators – cyclins, cyclin-dependent kinases (Cdks) and Cdk inhibitors – are frequently mutated in human cancer (Malumbres and Barbacid, 2001).

In normal cells, pRb proteins repress the transcription of genes required for DNA replication or mitosis and maintain cells in a quiescent state. This function is achieved through the sequestering of inactive E2F transcription factors and through the binding to histone deacetylases and chromatin remodeling complexes (Attwooll *et al*., 2004; Korenjak and Brehm, 2005; Macaluso *et al*., 2006). Upon mitogenic stimuli, D-type cyclins are induced and activate the cell-cycle kinases Cdk4 and Cdk6. Cyclin D-Cdk4/6 complexes phosphorylate and partially inactivate pRb, allowing the expression of some E2F target genes such as cyclin E. Induction of cyclin E allows the activation of Cdk2, which is also able to further phosphorylate and completely inactivate pRb, triggering the massive transcription of genes required for DNA replication and mitosis. Cdk2 is also able to bind A-type cyclins during S phase, whereas the control of G_2 and M phases mainly depends on cyclin A- and cyclin B-Cdk1 complexes (Sherr, 2000; Malumbres and Barbacid, 2005).

Mitogenic stimuli induce cyclins and therefore activate Cdks, whereas antimitogenic signals arrest this process by inducing members of the two families of Cdk inhibitors (CKIs), the INK4 and Cip/Kip families (Sherr and Roberts, 1999). INK4 proteins (p16^{INK4a}, p15^{INK4b}, p18^{INK4c} and p19^{INK4d}) specifically bind Cdk4 or Cdk6 proteins disturbing their binding to D-type cyclins and forcing a kinase inactive state. Cip/Kip inhibitors (p21^{Cip1}, p27^{Kip1} and p57^{Kip2}), on the other hand, are able to bind Cdk-cyclin complexes forming ternary structures. CKIs are induced in response to various antimitogenic stimuli. For instance, p21^{Cip1} has been shown to play a major role in inducing p53-dependent G_1 cell-cycle arrest following DNA damage, and it is also induced by transforming growth factor-β, along with p15^{INK4b}, resulting in Cdk inactivation (Malumbres and Carnero, 2003). Specific CKIs are also involved in senescence responses and the ageing-dependent control of proliferation in stem cells and specific progenitors (Janzen *et al*., 2006; Krishnamurthy *et al*., 2006; Molofsky *et al*., 2006). In culture, primary somatic cells undergo a finite number of divisions before they arrest

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as senescence cells (Hayflick, 1965). This cellular senescence is accompanied by high levels of Cdk inhibitors including p21^{Cip1}, p16^{INK4a} and p15^{INK4b} (Noda *et al*., 1994; Alcorta *et al*., 1996; Palmero *et al*., 1997; Stein *et al*., 1999). Similarly, these CKIs are induced in the premature senescence phenotype in response to inappropriate oncogenic stimulation. Thus, overexpression of oncogenic Ras in primary cells results in high levels of p16^{INK4a} (Serrano *et al*., 1997) and p15^{INK4b} (Malumbres *et al*., 2000), which are accompanied by p19^{ARF} induction and consequent activation of the p53/p21^{Cip1} pathway (Serrano *et al*., 1997; Palmero *et al*., 1998). In fact, overexpression of several INK4 and Cip/Kip proteins is sufficient to arrest cells in G_1 and induce a senescent-like phenotype (Serrano *et al*., 1997; McConnell *et al*., 1998; Vogt *et al*., 1998; Malumbres *et al*., 2000).

Although all these inhibitors participate in the senescence signaling pathways, individual inactivation of specific CKIs do not completely preclude senescence responses. Mice deficient in either p16^{INK4a}, p15^{INK4b}, p18^{INK4c} or p21^{Cip1} have only a limited susceptibility to tumor development in aged mice (Latres *et al*., 2000; Krimpenfort *et al*., 2001; Martin-Caballero *et al*., 2001; Sharpless *et al*., 2001). In addition, mouse embryonic fibroblasts (MEFs) without either of these inhibitors arrest in a senescence-like crisis period when cultured *in vitro* and are mostly resistant to transformation by Ras oncogenes (Pantoja and Serrano, 1999; Latres *et al*., 2000; Krimpenfort *et al*., 2001; Sharpless *et al*., 2001). The 'weak' phenotype of p16^{INK4a} and p15^{INK4b} individual mutants could be at least partially explained by compensation between these two inhibitors given the strong functional similarity between them. A double p16^{INK4a}-p15^{INK4b} mutant has not been reported yet since the corresponding genes lie only 12 kb apart in the mouse genome. However, the cooperative effect between these mutations has been investigated using a Cdk4 mutant (Cdk4^{R24C}) protein that is not inhibited by the INK4 proteins (Wolfel *et al*., 1995). Knock-in cells carrying this Cdk4^{R24C} mutant display proliferative advantages *in vitro* although they still display significant senescence-like features and resistance to cellular transformation (Sotillo *et al*., 2001a; Rane *et al*., 2002). Furthermore, genetic ablation of p21^{Cip1} in the mouse results in decreased DNA damage responses but not alter immortalization or susceptibility to Ras-induced transformation of primary fibroblasts (Brugarolas *et al*., 1995; Pantoja and Serrano, 1999). Similarly, disruption of p21^{Cip1} in diploid human fibroblasts by two sequential rounds of targeted homologous recombination bypass G_1 arrest allowing extended lifespan, but not immortalize targeted cells (Brown *et al*., 1997).

To investigate the relative roles of INK4 and Cip1 inhibitors in cellular senescence and malignant transformation, we have analysed the synergistic effect of combining the insensitivity to INK4 proteins and p21^{Cip1} deficiency. Overexpression of Cdk4^{R24C}, but not other cell-cycle regulators, efficiently rescues p21^{Cip1}-*null* cultured cells from senescence-like arrest. Genetic combination of these two alterations in Cdk4^{R24C} knock-in, p21^{Cip1}-*null* (Cdk4$^{R/R}$; p21$^{-/-}$) cells results in cellular immortality and high susceptibility to transformation by Ras oncogenes. Finally, Cdk4$^{R/R}$; p21$^{-/-}$ mice display increased tumor susceptibility than single mutants, suggesting a significant cooperative effect of deregulating both p21^{Cip1} and INK4 pathways in tumor progression.

Results

Overexpression of Cdk4^{R24C} rescues p21^{Cip1}-null *cells from senescence-like arrest*

To identify molecules that cooperate with p21^{Cip1} in triggering antiproliferative responses, we first overexpressed several wild-type and mutant cell-cycle regulators in p21^{Cip1}-deficient (p21$^{-/-}$) MEFs at passage 5, immediately preceding the senescence-like state of primary MEFs in culture. As expected, wild-type or p21-*null* MEFs are not efficient in bypassing the crisis period (Figure 1a). p21$^{-/-}$ cells show a slight advantage in the colony formation assay (22 colonies versus 2 colonies in wild-type cells). However, this proliferative advantage is clearly limited as cells expressing a dominant negative p53 mutant (p53 R175H) form 200 colonies in wild-type cells and more than 500 colonies in p21$^{-/-}$ cells. Among the other molecules tested, only the Cdk4 R24C (Cdk4^{R24C}) mutant displayed a significant activity in this assay. In fact, expression of Cdk4^{R24C} confers a proliferative advantage to p21^{Cip1}-*null*, but not wild-type cells, comparable to the effect of dominant negative forms of p53 (Figures 1a and b). Overexpression of other cell-cycle regulators such as cyclin D1, cyclin A1, cyclin E1, E2F1, E2F4 or Cdc25A did not rescue proliferation in wild-type or p21^{Cip1} mutant cells (Figure 1a). These results indicate that enforced expression of Cdk4^{R24C} is not sufficient to confer resistance to senescence-like arrest of wild-type cells, but efficiently cooperates with p21^{Cip1} deficiency to bypass this crisis period in cultured MEFs.

We then analysed the long-term proliferation rates of pooled cell populations in which we express ectopic Cdk4^{R24C} in primary wild-type or p21$^{-/-}$ MEFs, and compared these results to cells expressing the p53 dominant negative mutant (Figure 1c). Following a 3T3 protocol, wild-type or p21^{Cip1}-*null* cells do not proliferate beyond passage 6 (about 10–12 population doublings (PD)), although they resume growth approximately at passage 10 (PD 20–24). Ectopic overexpression of Cdk4^{R24C} results in earlier exit from the crisis period, indicating that this mutant contributes to bypass senescence-like arrest but is not sufficient to immortalize cells. In contrast, expression of Cdk4^{R24C} in p21^{Cip1}-*null* cells completely eliminates the crisis period resulting in a continuous and exponential growth (Figure 1c). Since the only effect of the R24C mutation in Cdk4 is to impede binding of the INK4 inhibitors (Wolfel *et al*., 1995), these results suggest that elimination of the cell-cycle control by INK4 and p21^{Cip1} is sufficient to avoid the culture crisis period and to immortalize primary MEFs in culture.

a

Gene	p21$^{+/+}$	p21$^{-/-}$
Vector	2 ± 2	22 ±14
p53^{R175H}	206 ± 54	> 500
Cdk4^{R24C}	42 ± 13	> 500
Cyclin D1	4 ± 3	20 ± 12
Cyclin A1	3 ± 2	12 ± 6
Cyclin E1	1 ± 1	10 ± 9
E2F1	0	21 ± 4
E2F4	0	9 ±5
Cdc25A	5 ± 3	8 ± 6

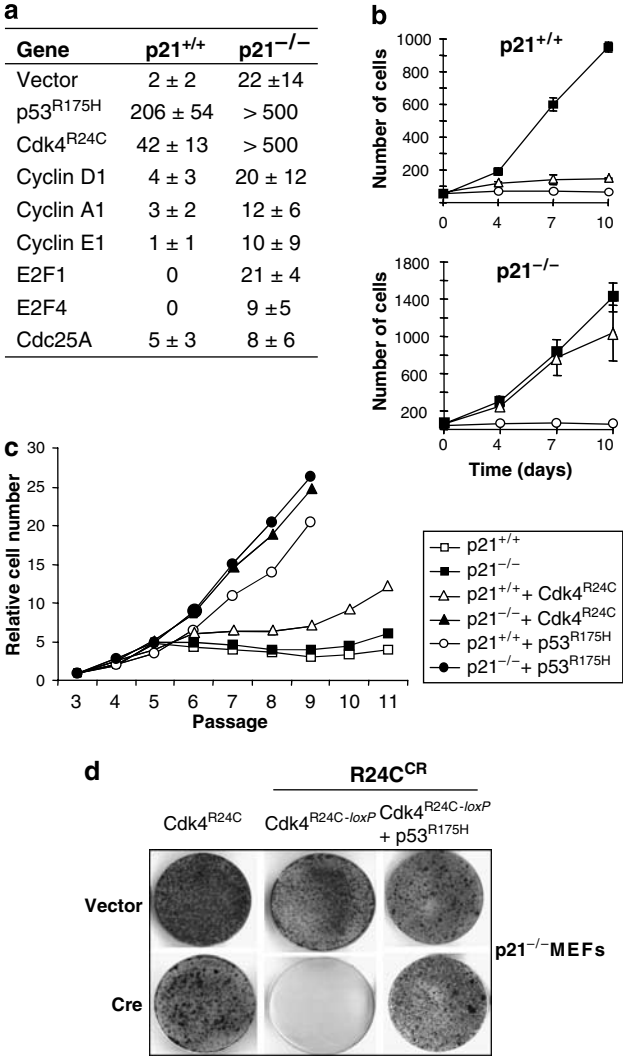

Figure 1 Cdk4R24C rescues growth arrest p21^{Cip1}-*null* MEFs. (**a**) Effect of the indicated cell-cycle regulators on the number of colonies formed in wild-type or p21^{Cip1}-*null* early-passage primary MEFs. The average ± s.d. after three separate experiments is shown. (**b**) Effect of Cdk4^{R24C} (open triangle), p53^{R175H} (filled squares) or the empty vector (open circles) on cell proliferation in wild-type or p21^{Cip1}-*null* cells. (**c**) Cell proliferation of wild-type or p21^{Cip1}-null MEFs after infection with the indicated molecules. The standard 3T3 passage protocol was followed as indicated in Materials and methods. (**d**) Cdk4^{R24C} expression is continuously required for proliferation of p21^{Cip1}-*null* cells that have been immortalized in the presence of this INK4-insensitive mutant protein. Cre-mediated deletion of the *loxP*-conditional Cdk4^{R24C} cDNA results in no colonies, a defect rescued by overexpressing the p53^{R175H} dominant negative mutant. Cdk, cyclin-dependent kinase; MEFs, mouse embryonic fibroblasts.

Dependency on Cdk4^{R24C} to maintain growth advantages

We next sought to test whether continuous inactivation of INK4 protein function was required for immortalization. For these experiments, we cloned the Cdk4^{R24C} mutant into pMarx vectors (Hannon *et al*., 1999), a retroviral vector that contains a Cre recombinase target site (loxP site). Upon integration of the retrovirus into the genome, the *loxP* site is duplicated such that the genes carried by the virus are flanked on either side by *loxP* sites. Subsequent expression of Cre recombinase causes excision at these *loxP* sites, leading to removal from the genome and eventual loss of expression of the exogenous gene (Carnero *et al*., 2000). Presenescent p21^{Cip1}-*null* MEFs were infected with the Cdk4^{R24C} mutant in pMarx and immortal cell lines, R24C^{CR}, were generated using a 3T3 protocol. At PD 32, R24C^{CR} cells were infected with a Cre-expressing virus to ablate Cdk4^{R24C} expression. Cells in which INK4 function had been restored failed to form colonies, while control cells continued to proliferate (Figure 1d). Immortalized cell lines that had been generated following infection with non-excisable Cdk4^{R24C}-expressing viruses did not arrest following Cre recombinase expression. Finally, expression of dominant negative p53 from a non-excisable vector in the R24C^{CR} reversible cell line overcame the arrest induced by excision of the Cdk4^{R24C} mutant construct (Figure 1d), indicating that the recovery of mortality could be bypassed by another immortalizing signal such as p53 inactivation. Together, these data indicate that INK4 proteins are still functional in the Cdk4^{R24C}-expressing cells after many passages and they are able to arrest cells if the overexpressed Cdk4^{R24C} mutant is eliminated.

Genetic cooperation between Cdk4^{R24C} and p21^{Cip1} deficiency

To characterize the synergistic effect of p21^{Cip1} and INK4 proteins *in vivo*, we took advantage of Cdk4 R24C (Cdk4$^{R/R}$) knock-in mice, which express endogenous levels of the Cdk4^{R24C} mutant (Rane *et al*., 1999). p21^{Cip1}-deficient mice (Brugarolas *et al*., 1995) were crossed with Cdk4$^{R/R}$ knock-in mice and primary MEFs were isolated from various genotypes. We next analysed the proliferation rates of wild-type (Cdk4$^{+/+}$; p21$^{+/+}$), single mutant (Cdk4$^{R/R}$; p21$^{+/+}$ or Cdk4$^{+/+}$; p21$^{-/-}$) and double mutant (Cdk4$^{R/R}$; p21$^{-/-}$) MEFs. As depicted in Figure 2a, deficiency in p21^{Cip1} (Cdk4$^{+/+}$; p21$^{-/-}$ cells) or expression of the Cdk4 R24C mutant (Cdk4$^{R/R}$; p21$^{+/+}$ cells) confers a relative proliferative advantage to primary MEFs in the early passages. At this stage, both mutations do not show any synergistic effect since proliferation of double mutant cells is similar to wild-type cells expressing the Cdk4 R24C mutant. However, as cells approach the crisis period, the differences are more evident. At passage 5, wild-type MEFs have entered crisis and have lost their capacity to duplicate the populations in culture (population doubling levels (PDLs) = 1.1). p21$^{-/-}$ or Cdk4$^{R/R}$ single mutant MEFs show a partial reduction in proliferative potential (PDL = 2.6 and 1.6, respectively). However, double mutant Cdk4$^{R/R}$; p21$^{-/-}$ cells do not show any reduction in the PD capacity and even increase their proliferative potential during passages 4–7 (Figure 2b). Accordingly, wild-type, Cdk4$^{R/R}$; p21$^{+/+}$ and Cdk4$^{+/+}$; p21$^{-/-}$ cells displayed morphological changes characteristic of senescent cells such as flat morphology and enlarged cellular bodies and nuclei (Figure 2c). However, these senescent-like features were barely visible in the actively

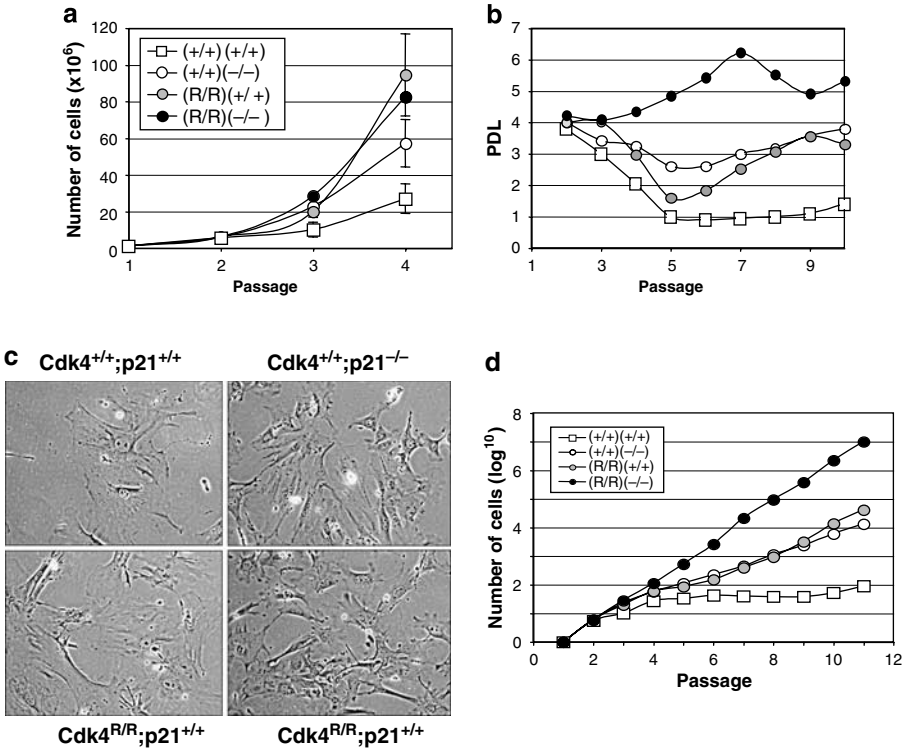

Figure 2 Genetic cooperation between Cdk4^{R24C} and p21^{Cip1}-deficiency in immortalization. Primary MEFs were isolated from wild-type ((+/+)(+/+); empty squares); Cdk4^{R24C} ((R/R)(+/+); gray circles), p21^{Cip1}-*null* ((+/+)(−/−); empty circles) and double mutant ((R/R)(−/−); filled circles) embryos and cultured following a standard 3T3 protocol. (**a**) Cell proliferation of early-passage MEFs with the different genotypes. (**b**) Population doubling levels (PDLs) of wild-type MEFs during the crisis period. Double mutant cells do not display any decrease in PDLs while single mutants show an intermediate phenotype. (**c**) Representative pictures of cultures of the different genotypes at passage (P) 5. (**d**) Immortalization of the different MEFs after the 3T3 protocol. Double mutants display no crisis period while single mutants display intermediate phenotypes. Cdk, cyclin-dependent kinase; MEFs, mouse embryonic fibroblasts.

proliferating Cdk4$^{R/R}$; p21$^{-/-}$ population, which do not show detectable crisis period and behavior as immortal cells (Figure 2d).

To obtain some insights into the molecular mechanism behind this cooperation, we analysed several cell-cycle regulators in primary MEFs with specific genetic alterations. p21^{Cip1}-*null* MEFs do not display major alterations in the levels of cell-cycle regulators such as Cdk2, Cdk4, Cdk1, A-type cyclins or the INK4 inhibitors (Figure 3a). Total protein levels of D-type cyclins were reduced in p21^{Cip1}-*null* cells as described previously (Cheng *et al*., 1999). This reduction is not due to decreased transcription (since mRNA levels are not reduced; data not shown) but to protein degradation as reported previously (Bagui *et al*., 2003). p27^{Kip1} is also slightly increased in p21-*null* (and Cdk4$^{R/R}$; p21$^{-/-}$) cells (Figure 3a). Kinase activities of Cdk2 and Cdk1 are not significantly altered in single or double mutant MEFs. However, at specific cell passages, Cdk4 kinase activity seems to be slightly decreased in Cdk4$^{+/+}$; p21$^{-/-}$ cells (Figure 3b), as it has been observed previously (Cheng *et al*., 1999). Binding of p27^{Kip1} to Cdk4 is not altered in these mutant primary MEFs. The fact that Cdk4^{R24C} is insensitive to INK4 proteins might therefore relief these putative side effects of p21^{Cip1} absence on Cdk4 activity.

Increased oncogenic susceptibility in the absence of INK4 and Cip1 pathways

Since immortalization is a prerequisite for cell transformation, we next analysed whether the combined alteration of Cdk4 and p21^{Cip1} in these double mutant MEFs cooperates in oncogenic transformation. Transfection of wild-type primary MEFs with oncogenic Ha-Ras does not result in foci formation, which is only achieved using a combination of Ras and E1A in these cells. As described previously (Sotillo *et al*., 2001a), Cdk4$^{R/R}$ MEFs display a slightly increased susceptibility to oncogenic transformation by Ras in this assay, similarly to that of Cdk4$^{+/+}$; p21$^{-/-}$ cells (Figure 4a). Interestingly, Cdk4$^{R/R}$; p21$^{-/-}$ double mutant MEFs display a dramatic increase in cellular transformation by Ras oncogenes (about 97 foci per assay versus 0 in wild-type cells and 4–9 foci in single mutants; Figure 4a). Similarly, double mutant MEFs also display a dramatic increase in the susceptibility to oncogenic transformation by a combination of Ras and E1A oncogenes (about 150 foci in double mutant MEFs versus 18–28 foci in single mutants and 5 foci in wild-type cells). To discard the possibility that Cdk4$^{R/R}$; p21$^{-/-}$ double mutant cells are already transformed, we analysed the growth of these and other control cells in soft agar. As

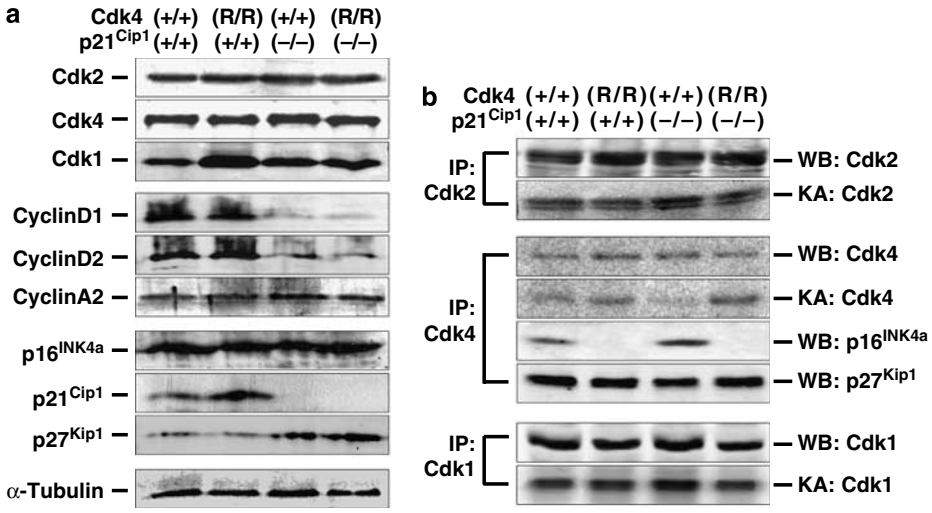

Figure 3 Cell-cycle regulators in Cdk4^{R24C} and p21^{Cip1} mutant mice. Protein lysates were obtain from wild-type ((+/+)(+/+)); Cdk4^{R24C} ((R/R)(+/+)), p21^{Cip1}-*null* ((+/+)(−/−)) and double mutant ((R/R)(−/−)) MEFs at P5. (**a**) Total protein levels for the indicated cell-cycle regulators. (**b**) Protein lysates were immunoprecipitated (IP) with antibodies against the indicated kinases. The amount of kinase immunoprecipitated and the binding to specific partners was evaluated by western blotting (WB). Kinase activity (KA) was tested in these immunoprecipitates using pRb (Cdk4) or histone 1 (Cdk2 and Cdk1) as substrates. Cdk, cyclin-dependent kinase; MEFs, mouse embryonic fibroblasts; P5, passage 5; pRb, retinoblastoma protein.

indicated in Figures 4b and c, none of the indicated genotypes, including Cdk4$^{R/R}$; p21$^{-/-}$ cells, formed colonies in soft agar, unless these cells were previously transfected with Ras and E1A oncogenes. Thus indicating that Cdk4$^{R/R}$; p21$^{-/-}$ cells, although immortal, are not transformed.

Cooperation between p21^{Cip1} and INK4 tumor suppressor pathways in vivo

Crosses between p21$^{-/-}$ and Cdk4$^{R/R}$ single mutant mice resulted in the expected ratio of Cdk4$^{R/R}$; p21$^{-/-}$ double mutant mice (data not shown). Young Cdk4$^{R/R}$; p21$^{-/-}$ mice present a phenotype similar to that described for Cdk4$^{R/R}$; p21$^{+/+}$ mice, including increased size and hyperplasia of some endocrine tissues such as the pancreatic endocrine islets (Rane *et al*., 1999). Both Cdk4$^{R/R}$; p21$^{+/+}$ and Cdk4$^{+/+}$; p21$^{-/-}$ single mutant mice display certain susceptibility to tumor development as reported previously (Martin-Caballero *et al*., 2001; Sotillo *et al*., 2001a; Rane *et al*., 2002). Spontaneous tumors develop in these animals with long latency, starting at 9 months (Cdk4$^{+/+}$; p21$^{-/-}$) or 12 months (Cdk4$^{R/R}$; p21$^{+/+}$) of age (Figure 5a). However, by 8–10 weeks, about 15% (5/33) of double mutants die. In all these cases, these animals displayed very long incisor teeth that prevent them from food uptake (data not shown). This feature was also observed in a Cdk4$^{R/R}$; p21$^{+/-}$ mouse and was not observed in any of the control animals. Whether this phenotype suggests neurological disorders or increased teeth formation is unknown at this moment. In addition to these few animals, additional 15% of Cdk4$^{R/R}$; p21$^{-/-}$double mutant mice die earlier than single mutant mice due to a variety of tumors. As indicated in Figure 5a, the average lifespan of Cdk4$^{R/R}$; p21$^{-/-}$ mice is of 48.5 weeks (versus 65 weeks in Cdk4$^{+/+}$; p21$^{-/-}$ and 68 weeks in Cdk4$^{R/R}$; p21$^{+/+}$ mice). Macroscopic and histologic examination of pathologies in these animals indicated the presence of a variety of tumors in these mutant mice (Table 1). Most of tumors that develop in Cdk4$^{R/R}$; p21$^{-/-}$ mice are also present in any of the single mutant mice suggesting that cooperation between these two mutations results in a shorter latency rather than new pathologies. The only exception is the presence of a significant increase in osteogenic tumors (11% incidence) develop in the skull of Cdk4$^{R/R}$; p21$^{-/-}$ double mutant mice but no the other genotypes (Table 1 and Figure 5). In addition to these osteosarcomas, the most frequent tumors in double mutant mice include angiosarcomas, and endocrine pathologies (neoplasis of the Leydig and pancreatic beta cell, and pituitary tumors), in all cases with similar incidence than in Cdk4$^{R/R}$; p21$^{+/+}$ mice (Table 1).

Discussion

Unscheduled proliferation in cancer cells frequently results as a consequence of hyperactivation of cell-cycle Cdks through amplification, mutation or overexpression of cyclins and, more frequently, inactivation of Cdk inhibitors (Malumbres and Barbacid, 2001). In fact, inactivation of the INK4 proteins is a frequent oncogenic alteration in several tumor types including sarcomas, lung, liver, pancreatic and hematopoietic malignancies (Malumbres and Barbacid, 2001). Moreover, p27^{Kip1} downregulation by increased proteolysis is a common event in tumor cells and correlates with poor prognosis. Downregulation of p21^{Cip1}, on the other hand, frequently results from inactivation of the p53 pathway (Malumbres and Carnero, 2003). In addition,

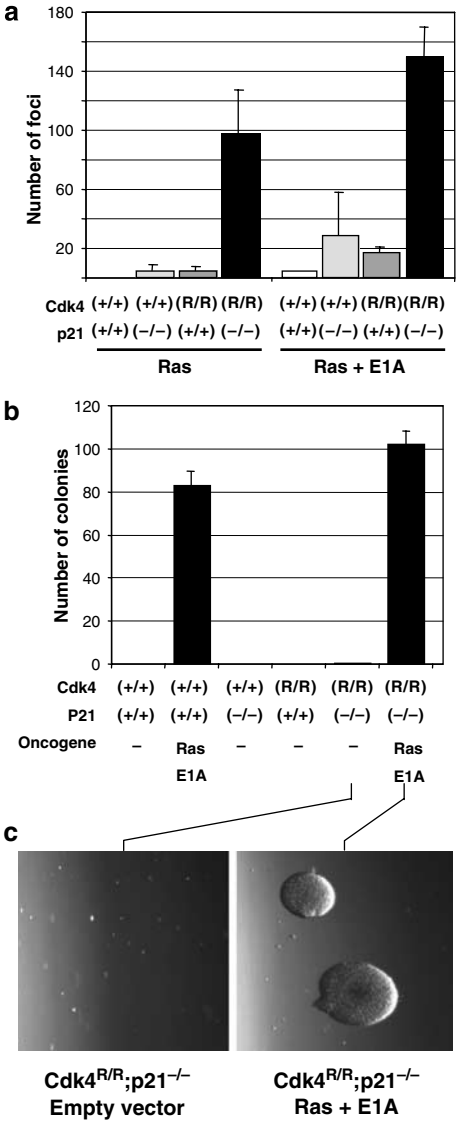

Figure 4 Double Cdk4$^{R/R}$; p21$^{-/-}$ MEFs are highly sensitive to cellular transformation. (**a**) Focus formation assay by Ras or Ras + E1A oncogenes in early-passage MEF with the different genotypes. (**b**) Growth of selected clones in soft agar and (**c**) representative pictures. Only clones transfected with Ras + E1A display significant growth in soft agar indicating that non-transfected Cdk4$^{R/R}$; p21$^{-/-}$ clones do not show oncogenic properties despite their immortal phenotype. Cdk, cyclin-dependent kinase; MEFs, mouse embryonic fibroblasts.

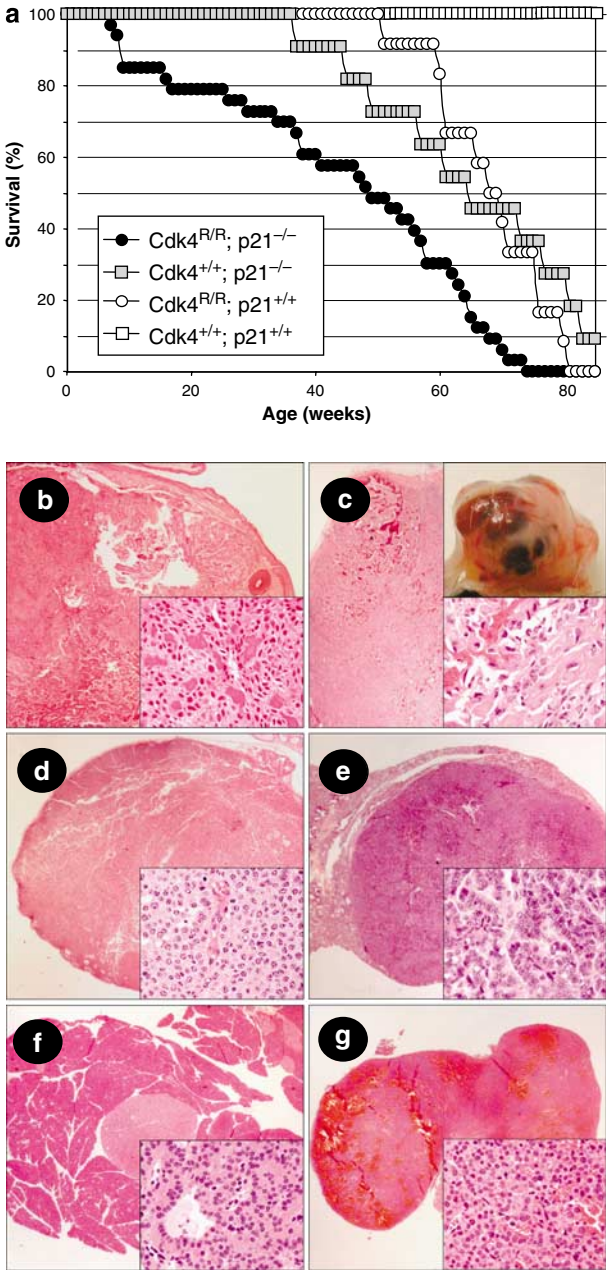

Figure 5 Survival and tumor development in Cdk4$^{R/R}$; p21$^{-/-}$ mice. (**a**) Survival of mice with different combinations of Cdk4 and p21^{Cip1} alleles as indicated (Cdk4$^{+/+}$; p21$^{+/+}$ ($n=25$); Cdk4$^{R/R}$; p21$^{+/+}$ ($n=12$); Cdk4$^{+/+}$; p21$^{-/-}$ ($n=11$) and Cdk4$^{R/R}$; p21$^{-/-}$ ($n=33$)). Microscopic images of hematoxylin and eosin-stained sections are shown (× 120 with insert at × 4000): (**b**) cementifying fibroma, (**c**) osteosarcoma, (**d**) Leydig cell tumor (testis), (**e**) lung adenocarcinoma, (**f**) pancreatic endocrine tumor and (**g**); pars distalis adenoma (pituitary). A macroscopic image of the osteosarcoma is also included in (**c**). Cdk, cyclin-dependent kinase.

this protein can be downregulated in tumor cells by epigenetic alterations in its promoter (Duan *et al*., 2005). The functional specificity of these two families of Cdk inhibitors has been analysed by different biochemical and genetic means. INK4 proteins specifically bind to monomeric Cdk4 or Cdk6 kinases (Sherr and Roberts, 1999). Genetic studies have shown that INK4 inhibitors do not arrest cell cycle in Cdk4/Cdk6-deficient cells, suggesting a strict functional correspondence between INK4 proteins and the inhibition of Cdk4 and Cdk6 kinase activities (Malumbres *et al*., 2004). Cip/Kip proteins bind all Cdk-cyclin complexes although it has been proposed that Cdk2 is the preferred target for inhibition. In addition, p21^{Cip1} and p27^{Kip1} do not inhibit Cdk4/6 kinase activity in certain circumstances and seem to participate in the stabilization of Cdk4/6-cyclin D complexes (Sherr and Roberts, 1999). However, additional Cdk-independent functions appear to contribute to tumor suppressor activities of p21^{Cip1}

Table 1 Tumor susceptibility in Cdk4^{R24C} and p21^{Cip1} mutant mice[a]

Tumor type	*Cdk4$^{+/+}$; p21$^{+/+}$* (n = *23*)	*Cdk4$^{R/R}$; p21$^{+/+}$* (n = *17*)	*Cdk4$^{+/+}$; p21$^{-/-}$* (n = *18*)	*Cdk4$^{R/R}$; p21$^{-/-}$* (n = *19*)
Angiosarcoma	0 (0%)	10 (59%)	1 (6%)	9 (47%)
Osteosarcoma	0 (0%)	0 (0%)	0 (0%)	2 (11%)
Histiocytic sarcomas	0 (0%)	1 (6%)	9 (50%)	0 (0%)
Squamous cell	0 (0%)	0 (0%)	1 (6%)	1 (5%)
Lung	0 (0%)	1 (6%)	1 (6%)	2 (11%)
Pancreatic endocrine	0 (0%)	6 (35%)	0 (0%)	4 (21%)
Pituitary	0 (0%)	3 (18%)	0 (0%)	4 (21%)
Leydig cell	0 (0%)	9 (53%)	0 (0%)	5 (26%)
Lymphoid	1 (4%)	0 (0%)	3 (17%)	1 (5%)
Total incidence	1 (4%)	16 (94%)	14 (78%)	100%
Average latency (weeks)	>90	69	64.5	53.7

Abbreviation: Cdk, cyclin-dependent kinase. [a]Figures indicate the number of animals that developed each specific tumor type and the corresponding incidence (%) for each genotype.

(Coqueret, 2002, 2003; Gregory *et al*., 2002). In fact, p21^{Cip1} is able to efficiently arrest the cell cycle in response to several antimitogenic signals in Cdk2-*null* cells, indicating that Cdk2 is not required for p21^{Cip1} tumor suppressor activity (Martin *et al*., 2005).

The tumor suppressor activity of INK4 and Cip1 proteins has been evaluated *in vivo* using different genetic models in the mouse. Deletion of p21^{Cip1} results in altered response to DNA damage responses and increased tumor susceptibility, specifically in mesenchymal and hematopoietic cells, at an advanced age (Brugarolas *et al*., 1995; Martin-Caballero *et al*., 2001). Absence of p21^{Cip1} accelerates the development of some tumors such as breast tumors in Ras-transgenic mice (Adnane *et al*., 2000; Bearss *et al*., 2002) and intestinal tumors in Apc-haploinsufficient mice (Yang *et al*., 2001), but has no effect on breast or lymphoid malignancies induced by Myc (Bearss *et al*., 2002; Martins and Berns, 2002). In addition, p21^{Cip1} deficiency has contradictory effects on thymic lymphomas and skin tumors (Wang *et al*., 1997; Philipp *et al*., 1999; Topley *et al*., 1999; Weinberg *et al*., 1999; De la Cueva *et al*., 2006). Ablation of individual members of the INK4 family similarly results in limited oncogenic phenotype, mostly restricted to reduced susceptibility to tumor development in old p16^{INK4a} or p18^{INK4c}-*null* mice (Franklin *et al*., 1998; Latres *et al*., 2000; Zindy *et al*., 2000; Krimpenfort *et al*., 2001; Sharpless *et al*., 2001). The cooperation between Cip/Kip proteins and INK4 inhibitors has been evaluated using mice deficient in p18^{INK4c} in a p21^{Cip1}- or p27^{Kip1}-*null* background (Franklin *et al*., 2000). Loss of both p18^{INK4c} and p21^{Cip1} results in specific cooperation in pituitary and lung tumors whereas combined ablation of p18^{INK4c} and p27^{Kip1} significantly increases endocrine (pituitary, adrenals, thyroid, testes and pancreas) and gut tumors (Franklin *et al*., 2000). Inactivation of these Cip/Kip proteins has also been evaluated in a p16^{INK4a}/ARF-*null* background. p27^{Kip1} deficiency in this background results in acceleration of T-cell lymphomas whereas, in contrast, deficiency in p21^{Cip1} produce no overt alteration in p16^{INK4a}/ARF-*null* mice (Martin-Caballero *et al*., 2004). Since these p16^{INK4a}/ARF-*null* mice display a partial inactivation of the p53 pathway owing to genetic ablation of p19^{ARF}, these results may reflect cooperation between p27^{Kip1}, but not p21^{Cip1}, and the ARF-p53 pathway.

Combined ablation of all the four members of this family has not been evaluated so far. In fact, the close proximity between p16^{INK4a} and p15^{INK4b} genes has prevented so far genetic crosses between knockout mice for these inhibitors. This limitation has been partially overcome by the use of knock-in mice that express Cdk4^{R24C} mutant proteins that are insensitive to all these INK4 inhibitors (Rane *et al*., 1999). These mice develop a wide spectrum of tumors with complete penetrance including epithelial, mesenquimal and lymphoid malignancies (Sotillo *et al*., 2001a, b; Rane *et al*., 2002). Our report provides genetic demonstration that combination of p21^{Cip1} and INK4 deficiencies (in the Cdk4^{R24C} background) significantly cooperates in development of mesenchymal tumors, and specifically those of bone origin (Figure 5 and Table 1).

The cooperation between Cip1 and INK4 proteins in mesenchymal cells agrees with the cooperative effect of these two pathways on the proliferative properties of fibroblasts in culture and their senescence responses. Senescence-like arrest of human and mouse fibroblasts in culture is mediated by p21^{Cip1} and members of the INK4 family such as p16^{INK4a} and p15^{INK4b} (Alcorta *et al*., 1996; Brown *et al*., 1997; Palmero *et al*., 1997; Serrano *et al*., 1997; McConnell *et al*., 1998; Stein *et al*., 1999; Malumbres *et al*., 2000). However, inactivation of individual Cdk inhibitors only partially relieves the senescence response (Medcalf *et al*., 1996; Pantoja and Serrano, 1999; Latres *et al*., 2000). We have shown in this report that expression of the INK4-insensitive Cdk4^{R24C} mutant efficiently rescues p21^{Cip1}-*null* cells from senescence. In addition, combined inactivation of Cip1 and INK4 pathways by genetic means eliminates the senescence response to culture shock in primary fibroblasts. Cdk4$^{R/R}$; p21$^{-/-}$ mutant cells behavior as immortal cells although they are not transformed. However, they are highly sensitive to transformation by single oncogenes such as Ras. Of note, pRb/p107/p130 triple knockout MEFs are refractory to transformation by Ras oncogenes (Dannenberg *et al*., 2000; Sage *et al*., 2000), suggesting that the cooperative effect of p21^{Cip1} deficiency in a Cdk4^{R24C} background transcends beyond the pRb pathway. In fact, combined alteration of Cdk4 and p21^{Cip1} equals p53 deficiency and further inactivation of p53 does not seem to cooperate in these assays (data not shown). Moreover, the cooperation between p21^{Cip1} deficiency and Cdk4^{R24C} results in increased susceptibility to tumor formation *in vivo*, at least in specific mesenquimal cells. Given the recent demonstration that senescence function as a tumor suppressor mechanism *in vivo* (Braig and Schmitt, 2006) and the involvement of INK4 inhibitors in age-dependent proliferation (Janzen *et al*., 2006; Krishnamurthy *et al*., 2006; Molofsky *et al*., 2006), these data

indicate that concomitant inactivation of p21^{Cip1} and INK4 inhibitors might help tumor cells to become insensitive to senescence-induced antiproliferative signals.

Materials and methods

Mice and histological analysis

Cdk4^{R24C} knock-in mice (Rane *et al*., 1999) and p21^{Cip1}-deficient animals (Brugarolas *et al*., 1995) have been reported previously. These animals were maintained in a mixed 129/Sv (25%) × CD1 (25%) × C57BL/6J (50%) background. Mice were housed at the pathogen-free animal facility of the Centro Nacional de Investigaciones Oncológicas (Madrid) following the animal care standards of the institution. These animals were observed in a daily basis and sick mice were euthanized humanely in accordance with the Guidelines for Humane End Points for Animals used in biomedical research. Tumor latency has been considered equivalent to lifespan. For histological observation, dissected organs were fixed in 10%-buffered formalin (Sigma-Aldrich, St Louis, MO, USA) and embedded in paraffin wax. Three- or five-micrometer-thick sections were stained with hematoxylin and eosin. Additional immunohistochemical examination of the pathologies observed was performed essentially as described in Sotillo *et al*. (2001a).

Cell culture

MEFs were prepared from E13.5 embryos using standard protocols (Sotillo *et al*., 2001a). Head and blood organs were removed, and the torso was minced and dispersed in 0.1% trypsin (20 min at 37°C). Cells were grown for two PD and then frozen. MEFs were subcultured 1:4 upon reaching confluence; each passage was considered to be two PDLs. All cultures were maintained in Dulbecco's modified Eagle's medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 2 mM glutamine, 1% penicillin/streptomycin and 10% fetal bovine serum (FBS) or donor calf serum.

Growth properties were analysed the classical 3T3 protocol. Every 3 days, cells were trypsinized, counted and 10^6 cells were plated per 10-cm plate. The relative number of cells is considered as a measure of the number of cells per passage related to the initial number of cells seeded per plate. DNA content was analysed by flow cytometry (Becton-Dickinson, Franklin Lakes, NJ, USA).

Colony formation assays and retroviral-mediated gene transfer

Wild-type MEFs normally undergo senescence in passages 5–6, after 10–12 PD. When seeded at passage 5 (PD 10) and cultured with FBS for additional 12 days, they fail to form colonies. To analyse the effect of different cell-cycle proteins in bypassing cellular senescence in this assay, we infected early passage presenescent MEFs with retrovirus carrying different cell-cycle regulators. Cells were drug-selected for provirus integration and grown until passage 5 (PD 10), and 10^5 cells were plated in 10-cm dishes and cultured for 12 days. For focus assays, primary MEFs were transfected with RasG12V and/or E1A-expressing vectors as described previously (Sotillo *et al*., 2001a). Cells were cultivated over 15 days, with medium changed every 3 days, before being fixed and stained with crystal violet.

For retroviral infections, LinXE cells (5×10^6) were plated in a 10-cm dish, incubated for 24 h and then transfected by calcium phosphate precipitation with 20 μg of the retroviral plasmid (16 h at 37°C). After 48 h, the virus-containing medium was filtered (0.45 μm filter; Millipore, Bedford, MA, USA) and supplemented with 8 μg/ml polybrene (Sigma) and an equal volume of fresh media. Target fibroblasts were plated at 8×10^5 cells per 10-cm dish and incubated overnight. For infection, the culture medium was replaced by the appropriate viral supernatant, and then the culture plates were centrifuged (1 h, 1500 r.p.m.) and incubated at 37°C for 16 h. Cultures were selected where indicated with 75 μg/ml hygromycin (Calbiochem, La Jolla, CA, USA), 400 μg/ml G418 (Sigma), 1 μg/ml blasticin (Sigma) or 2 μg/ml puromycin (Fluka, Hauppauge, NY, USA). For the analysis of growth, cells were infected as described above and, at PD 12, 3×10^3 cells were plated in 2.5-cm dishes. At 2–3 day intervals, cells were fixed and stained with crystal violet. After extensive washing, crystal violet was resolubilized in 10% acetic acid and quantified at 595 nm as a relative measure of cell number.

Immunoprecipitation, western blotting and kinase assays

Cells were washed twice with ice-cold phosphate-buffered saline and lysed in NP-40 lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris–HCl pH 8.0, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 25 μg/ml aprotinin, 1 mM ethylenediamine tetraacetic acid). After 30 min on ice, samples were vortexed (5 min at 4°C) and cleared by centrifugation. Proteins were separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), transferred to nitrocellulose membranes (Bio-Rad, Richmond, CA, USA), probed using specific antibody and detected using fluorescent donkey (Rockland, Gilbertsville, PA, USA) or goat (Invitrogen, Carlsbad, CA, USA) anti-rabbit secondary antibodies followed by detection using the Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE, USA). After transfer of the protein lysates, we probed nitrocellulose membranes with antibodies against Cdk2, Cdk1, Cdk4, cyclin D2, p16^{INK4a}, p21^{Cip1} and ERK (Santa Cruz Biotechnology, Santa Cruz, CA, USA), cyclin D1 (LabVision, Fremont, CA, USA), p27^{Kip1} (Transduction Laboratories, Lexington, KY, USA) and α-tubulin (Sigma). For immunoprecipitation, 500 μg of total proteins were incubated with 2 μg of antibody against Cdk2, Cdk4 or Cdk1 during 4 h at 4°C. Then, bound to protein A-sepharose during 1 h. Protein A-sepharose bound proteins were washed three times in lysis buffer, boiled and run using 12% PAGE. Detection of specific proteins was performed as before. Kinase assays were performed essentially as described previously (Martin *et al*., 2005). A total of 1 μg of mouse pRb protein fragment (amino acids 769–921; Santa Cruz Biotechnology) or histone H1 (Roche, Mannheim, Germany) were used as substrates.

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3.2.- Regulación del ciclo celular en el desarrollo de la hipófisis y sus enfermedades

La hipófisis regula diversas funciones fisiológicas, incluyendo el crecimiento, el metabolismo, la reproducción, la respuesta ante el estrés y el envejecimiento. Los primeros modelos de ratón modificados genéticamente que aparecieron nos mostraron que la hipófisis es un órgano muy sensible a alteraciones genéticas específicas de los reguladores de ciclo celular como la proteína del retinoblastoma (pRB) o el inhibidor de ciclo celular, $p27^{Kip1}$. El análisis molecular de gran número de neoplasias en hipófisis humanas ha corroborado que la desregulación del ciclo celular está implicada de manera significativa en la tumorigénesis de la hipófisis. En concreto, proteínas involucradas en la regulación de las quinasas dependientes de Ciclina (Cdks) o en la ruta de pRB se hayan alteradas en casi todos los tumores humanos de hipófisis. Otros reguladores de ciclo celular como PTTG1/securina podrían tener un papel fundamental en las neoplasias de hipófisis promoviendo la inestabilidad genómica. Los datos experimentales recientes sugieren que estos reguladores de ciclo celular podrían tener una implicación significativa en la biología de las putativas células progenitoras y la homeostasis de este órgano. La comprensión de cómo los reguladores de ciclo celular controlan la biología de la hipófisis nos podrían dar nuevas aproximaciones terapéuticas contra las enfermedades de la hipófisis.

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Contribución del doctorando al trabajo

Victor Quereda realizó el trabajo de documentación bibliográfica y colaboró en la escritura del artículo.

REVIEW

Cell cycle control of pituitary development and disease

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Abstract

The pituitary gland regulates diverse physiological functions, including growth, metabolism, reproduction, stress response, and ageing. Early genetic models in the mouse taught us that the pituitary is highly sensitive to genetic alteration of specific cell cycle regulators such as the retinoblastoma protein (pRB) or the cell cycle inhibitor $p27^{Kip1}$. The molecular analysis of human pituitary neoplasias has now corroborated that cell cycle deregulation is significantly implicated in pituitary tumorigenesis. In particular, proteins involved in cyclin-dependent kinase regulation or the pRB pathway are altered in nearly all human pituitary tumors. Additional cell cycle regulators such as PTTG1/securin may have critical roles in promoting genomic instability in pituitary neoplasias. Recent experimental data suggest that these cell cycle regulators may have significant implications in the biology of putative progenitor cells and pituitary homeostasis. Understanding how cell cycle regulation controls pituitary biology may provide us with new therapeutic approaches against pituitary diseases.

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Introduction

The pituitary gland is a central endocrine organ that regulates basic physiological functions including growth, reproduction, and metabolic homeostasis. The mammalian pituitary is composed of three lobes: the posterior pituitary (PP), the intermediate lobe (IL, atrophic in humans), and the anterior pituitary (AP). The versatile endocrine functions of the gland are carried out by six cell types residing in the AP and IL of the pituitary gland. These cell types are defined by the hormone they produce and secrete: corticotropes producing ACTH, thyrotropes secreting TSH, somatotropes secreting GH, lactotropes that produce prolactin, gonadotropes secreting LH, and FSH, and the IL-specific melanotropes secreting MSH (Fig. 1). The adult pituitary arises from progenitors of a neuroectodermic primordium known as Rathke's Pouch in a temporal and spatial-specific fashion during pituitary development (Melmed 2003, Zhu *et al.* 2007). By embryonic day (E)9·5, specific signaling gradients induce the formation of the Rathke's Pouch from the oral ectoderm. The major proliferation phase and the positional determination and lineage commitment of the pituitary take place by mid-gestation (E11·5–E13·5) and the gland is not terminally differentiated till birth. Major pathways implicated in the development of the pituitary include the Notch and Wnt regulatory networks, which are mainly active in the early phases of pituitary organogenesis and are essential for the emergence of somatotropes, lactotropes, and thyrotropes (Zhu *et al.* 2007). The regulation of the proliferative ability of pituitary cells in adulthood is not well established, although different classes of stem/progenitor cells have been postulated (Vankelecom 2007). A side population that efficiently excludes the Hoechst 33342 dye has been shown to segregate with sphere-forming cells in the pituitary (Chen *et al.* 2005). Pituitary colony-forming cells that display notable clonogenic potential have also been isolated (Lepore *et al.* 2005). More recently, stem-cell specific markers such as SOX2+, SOX9, or OCT4 in addition to other epithelial markers have been found in a single-cell layer in the marginal zone suggesting the presence of stem/progenitor cells that may contribute to cell renewal in the adult pituitary (Fauquier *et al.* 2008, Garcia-Lavandeira *et al.* 2008, Gleiberman *et al.* 2008).

Control of the cell cycle by cyclin-dependent kinases and their regulators

The cell cycle is the process by which cells divide into daughter cells. Cell division is traditionally divided into four phases: S phase (synthesis of DNA) in which is produced the duplication of the genome, M phase

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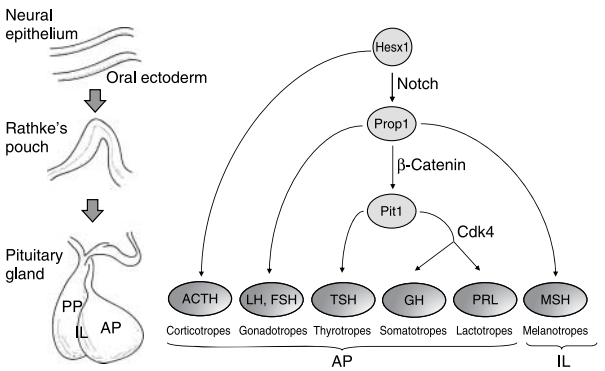

Figure 1 Development of pituitary and generation of hormone-producing cells from progenitors. Some representative transcription factors and signaling pathways are indicated. The cell cycle regulator CDK4 may be involved in the post-natal production of some AP cells such as somatotropes and lactotropes. The requirement for CDK4 in other pituitary cells is not clear as the whole pituitary is smaller in Cdk4-null mice. PP, posterior pituitary; IL, intermediate lobe; and AP, anterior pituitary.

(mitosis) in which the genetic material is segregated into two identical daughter cells, and two phases of growing and transition, called G (gap) phases (Fig. 2). G1 phase occurs before S phase; and G2 precedes mitosis. In mammalian cells, this process is driven by several protein kinases that regulate progression through the various phases of the cell cycle. Among these kinases, cyclin-dependent kinases (CDKs) are critical regulators of the transition through the different phases of the cell cycle (Malumbres & Barbacid 2005). CDK activity is modulated by fluctuations in the cellular concentration of their activators (cyclins) or inhibitors (CDK inhibitors or CKIs), which are regulated by specific transcriptional induction by mitogenic and anti-mitogenic pathways and proteolysis by the ubiquitin-proteosome system. A variety of cyclin and CDK complexes participate in the regulation of G1/S or G2/M transitions. D-type cyclins (D1, D2, and D3) act as sensors of multiple mitogenic signals to activate CDK4 and CDK6 and to facilitate the progression during G1. CDK2–cyclin E (E1 and E2) complexes become active at the end of G1 and participate in the transition from G1 to S phase. E-type cyclins are substituted by A-type cyclins (A1, A2) to activate CDK2 and CDK1 at the end of S phase and during G2. Finally, the mitotic complex formed of CDK1–cyclin B (mostly B1 and B2) is involved in the progression through G2 and entry into M phases.

The specific inhibitors of CDKs (CKIs) also play a major role in the cell cycle as mediators of antimitogenic signals or checkpoint responses. They counteract CDK function, either by blocking their activation, or by impairing substrate/ATP access. There are two families of CKIs, the INK4 family and the Cip/Kip family. The INK4 family ($p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$) inhibits progression through G1/S by binding CDK4 and CDK6. By contrast, members of the Cip/Kip family ($p21^{Cip1}$, $p27^{Kip1}$,and $p57^{Kip2}$) have different roles depending on the CDK–cyclin complex they bind to. Association to CDK2 and CDK1 complexes blocks their kinase activity, whereas the role of Cip or

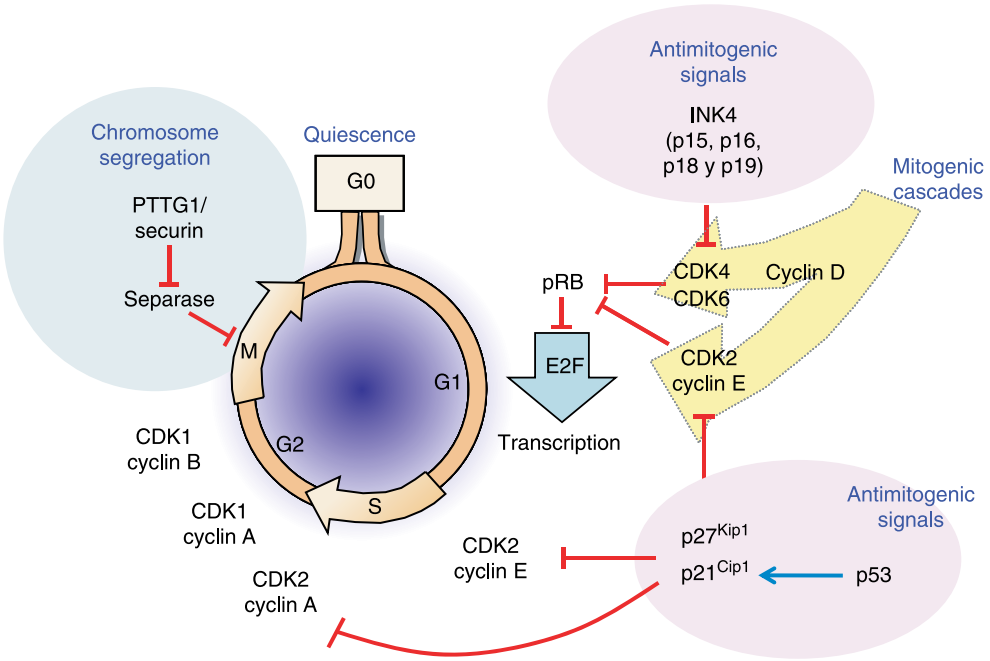

Figure 2 Control of the cell division cycle by major regulators involved in pituitary biology. S, DNA synthesis; M, mitosis; G1 and G2 correspond to 'gap' phases. Quiescence is frequently referred to as G0.

Kip binding to CDK4–cyclin D or CDK6–cyclin D complexes is unclear (Malumbres & Barbacid 2005).

The primary substrates of the CDKs in G1 progression are the members of the retinoblastoma protein family (pRB). pRB negatively regulates entry into the cell cycle and G1/S progression (Malumbres & Barbacid 2001). pRB binds to the transcription factor family E2F to target cell cycle-specific genes for repression. In non-cycling cells, pRB is hypo-phosphorylated and this active form is able to repress cell cycle progression. CDK–cyclin mediated phosphorylation of pRB provokes its release from E2F factors that are then active to induce the expression of cell cycle genes required for S and M phases.

Pituitary function and mouse models of cell cycle deregulation

Little is known about the implication of cell cycle regulators in pituitary gland development. However, in the last years, several mouse models of cell cycle regulators, such as pRB, CDKs, or CKIs, have suggested that some endocrine tissues such as the pituitary gland are critical targets of cell cycle deregulation in cancer and other diseases.

The initial link between cell cycle regulation and the pituitary comes from the seminal genetic analysis of pRB in the mouse (Clarke *et al.* 1992, Jacks *et al.* 1992, Lee *et al.* 1992). In contrast to humans, in whom individuals who inherit one defective copy of pRB gene have a roughly 90% likelihood of developing retinoblastoma at an early age (Matsunaga 1980), mice heterozygous for pRB did not develop retinoblastoma but instead developed pituitary tumors by the age of 12 months (Jacks *et al.* 1992; Table 1). Tumor incidence and histological phenotype of the tumors was highly dependent on the mouse strain suggesting additional modifier genes in pituitary tumor development (Leung *et al.* 2004). Tumor incidence provoked by the partial deletion of pRB is partially reverted by a mutation in pRB effectors such as E2f1 (Yamasaki *et al.* 1998) or E2f4 (Lee *et al.*

Table 1 Mouse models of cell cycle-related proteins involved in pituitary biology

Model	Pituitary phenotype	Incidence (%)	Latency (months)	References
Pituitary hyperplasia				
$pRb^{+/-}$	IL tumors	100	16	Jacks *et al.* (1992)
$pRb^{+/-}$; E2f-$1^{-/-}$	IL tumors. Decreased versus pRB mutants	62	18	Yamasaki *et al.* (1998)
$pRB^{+/-}$; E2f-$4^{-/-}$	IL tumors. Decreased versus pRB mutants	78	20	Lee *et al.* (2002)
$p27^{-/-}$	IL tumors	100	12	Kiyokawa *et al.* (1996) and Nakayama *et al.* (1996)
$p27^{CK-/CK-}$	IL tumors	75	10·7	Besson *et al.* (2007)
$p27^{-/-}$;$Cdk2^{-/-}$	IL tumors. No differences versus P27 $^{-/-}$;$Cdk2^{+/+}$	100	12	Martin *et al.* (2005)
$pRb^{+/-}$; p27 $^{-/-}$	Cooperation in IL tumors	90	7	Park *et al.* (1999)
$p18^{-/-}$	Tumors in IL and AP	50	15	Franklin *et al.* (1998)
$p16^{-/-}$; $p18^{-/-}$	IL tumors. Shorter latency versus p18 mutants	50	10	Ramsey *et al.* (2007)
$p15^{-/-}$; $p18^{-/-}$	No differences versus p18 mutants	50	15	Latres *et al.* (2000)
$p19^{-/-}$; $p18^{-/-}$	No differences versus p18 mutants	50	15	Zindy *et al.* (2001)
$p21^{-/-}$; $p18^{-/-}$	Cooperation in IL tumors	90	13	Franklin *et al.* (2000)
$p27^{-/-}$; $p18^{-/-}$	IL and AP undifferentiated tumors	100	3·5	Franklin *et al.* (1998)
$pRB^{+/-}$; $p21^{-/-}$	IL tumors	100	12	Brugarolas *et al.* (1998)
$Cdk4^{R24C/R24C}$	AP tumors	25	15	Rane *et al.* (2002) and Sotillo *et al.* (2001)
K5-Cdk4; p27 $^{-/-}$	Cooperation in IL tumors	100	3	Macias *et al.* (2008)
$Cdk4^{R24C/R24C}$; p27 $^{-/-}$	Strong cooperation and undifferentiated tumors	100	2	Sotillo *et al.* (2005)
$pRB^{+/-}$; $Pttg1^{-/-}$	IL tumors with decreased incidence versus pRB mutants	30	13	Chesnokova *et al.* (2005)
$pRB^{+/-}$; αGSU.PTTG1	Overexpression of securin cooperates in AP tumors	100	16	Donangelo *et al.* (2006)
Pituitary hypoplasia				
$Cdk4^{-/-}$	Defective proliferation and endocrine cell numbers	100	Postnatal	Rane *et al.* (1999)
$Securin^{-/-}$	Hypoplastic pituitary	ND	ND	Melmed (2003)

ND, Not determined.

2002), indicating the relevance of the pRB/E2F pathway in pituitary tumorigenesis. The sole overexpression of another E2F family member, E2f3, is not sufficient to produce pituitary tumors, although these transgenic mice develop pituitary hyperplasia (Lazzerini Denchi *et al.* 2005).

The genetic analysis of pRB in the mouse clearly demonstrated a tumor suppressor function for this protein, and specifically in endocrine organs such as the pituitary. By that time, pRB function in the cell cycle was not fully explored and the relationship with the pituitary was not obvious. More than 15 years later, the reasons for the special sensitivity of endocrine tissues and particularly the pituitary, to pRB lost are not understood yet. However, this close relationship is not restricted to pRB protein. In 1996, three groups reported multiple organ hyperplasia, including pituitary tumors in $p27^{Kip1}$ mutant mice (Fero *et al.* 1996, Kiyokawa *et al.* 1996, Nakayama *et al.* 1996). As in the pRB mutants, $p27^{Kip1}$-deficient mice developed pituitary tumors by the age of 12 months (Kiyokawa *et al.* 1996, Nakayama *et al.* 1996). Although, in both cases the animals developed IL tumors, they present differential patterns in both the histological phenotype and the gene profile expression (Chien *et al.* 2007). Soon after, a significant incidence of pituitary tumors was described in mice deficient in another cell cycle inhibitor, the member of the INK4 family $p18^{INK4c}$. Fifty percent of these animals developed aggressive pituitary tumors mostly from the IL by 15 months, although some tumors originated from the AP (Franklin *et al.* 1998). Deficiency in either of the other INK4 proteins, $p16^{INK4a}$, $p15^{INK4c}$,or $p19^{INK4d}$ does not result in pituitary tumors. However, genetic ablation of both $p16^{INK4a}$ and $p18^{INK4c}$ cooperates both in the incidence and the latency of the development of the pituitary tumors (median survival of 10 months; Ramsey *et al.* 2007). No cooperation in pituitary tumor suppression is observed between $p18^{INK4c}$ and $p15^{INK4b}$ (Latres *et al.* 2000) or $p19^{INK4d}$ (Zindy *et al.* 2001).

INK4 proteins specifically inhibit CDK4 and CDK6 kinases by competing with the obligate activator of these kinases, the cyclins. The relevance of INK4 proteins as key inhibitor of CDK4 and CDK6 is highlighted by a specific mutation in CDK4 (Arg24 to Cys) that prevents inhibition of this kinase by INK4 proteins. This mutation has been observed in both hereditary and spontaneous melanoma with low incidence (Malumbres & Barbacid 2001). When a Cdk4 R24C mutant protein is expressed in the mouse in substitution of the endogenous wild-type protein, these knock-in mice develop multiple tumors including frequent endocrine and mesenchymal tumors (Sotillo *et al.* 2001, Rane *et al.* 2002). Interestingly, pituitary tumors are also frequent (around 25% in all the studies) in these knock-in mice suggesting the relevance of CDK4 kinase activity in these neoplasias. Most of these pituitary tumors originated in the AP with an average latency of around 15 months.

One or several cell cycle pathways in pituitary tumorigenesis?

The former models suggest a clear relevance of the CDK (and their inhibitors INK4 or KIP)/pRB pathway in pituitary tumorigenesis. However, the results obtained from the combination of some of these mutations in the mouse suggest a more complex molecular network. The combined deletion of pRB and $p27^{Kip1}$ results in shorter latency of pituitary tumors in p27 (−/−); pRb (+/−) mice (Park *et al.* 1999). In addition, the expression of $p27^{Kip1}$ mRNA is reduced in pituitary tumors from pRb(+/−) mice, suggesting that $p27^{Kip1}$ downregulation is necessary for the tumorigenicity of the pituitary even in a pRb-null background. Similarly, although $p21^{Cip1}$-null mice do not develop pituitary tumors, this mutation cooperates with pRb mutation by decreasing the latency of pituitary tumors from 12 to 9 months (Brugarolas *et al.* 1998). Similarly, both $p27^{Kip1}$ and $p21^{Cip1}$ deficiency accelerates pituitary tumorigenesis in a $p18^{INK4c}$-null background (Franklin *et al.* 1998, 2000). This cooperation is dramatic in double $p27^{Kip1}$; $p18^{INK4c}$ mutants, which develop pituitary adenomas within 3 months (Franklin *et al.* 1998).

Since both INK4 and CIP/KIP proteins are CDK inhibitors, these results suggested that these molecules cooperate in tumor suppression by strongly inactivating CDK function in the pituitary (Fig. 3). INK4 proteins specifically inhibit CDK4/6 kinases, whereas CIP/KIP proteins seem to preferentially inhibit CDK2 and CDK1. In agreement with this model, no cooperation in pituitary tumor formation is observed in double Cdk4 R24C; p18-null mice (Sotillo *et al.* 2005). However, the introduction of the mutated Cdk4 R24C allele in a p27-null background dramatically accelerates the development of pituitary tumors that kill these mutant mice in 8–10 weeks (Sotillo *et al.* 2005). No cooperation in pituitary tumor development is observed in mice mutant for Cdk4 R24C and deficient in $p21^{Cip1}$ (Quereda *et al.* 2007). However, a dramatic cooperation in pituitary tumor development is observed in mutant mice carrying a combination of the Cdk4 R24C, p21-null, and P27-null alleles (V Quereda and M Malumbres, unpublished observations). These results, together with the cooperation observed between pRb and $p27^{Kip1}$ (Park *et al.* 1999), suggest the existence of two major pathways for G1/S phase deregulation in pituitary tumors. One branch is formed

of $p18^{INK4c}$/CDK4/pRB, whereas the other one is represented by $p27^{Kip1}$ and perhaps $p21^{Cip1}$ (Fig. 3).

The preference of CIP/KIP proteins for CDK family members other than CDK4/6 indicated that these inhibitors may target CDK2, the other interphase CDK involved in G1/S transition. However, $p27^{Kip1}$ deficiency provokes similar pituitary tumors in both Cdk2(+/+) and Cdk2(−/−) mice (Martin *et al.* 2005) indicating that CDK2 is dispensible for these tumors and it is therefore not the critical target of $p27^{Kip1}$. Whether the other major cell cycle protein, CDK1, is the critical target of $p21^{Cip1}$ or $p27^{Kip1}$ during pituitary tumor suppression has not been fully addressed yet.

The complexity in the molecular pathways involved in pituitary tumorigenesis has recently increased after a new mouse model that suggests possible oncogenic functions of $p27^{Kip1}$. In this model, the authors designed a $p27^{Kip1}$ mutant allele that does not bind cyclins and CDKs and is mostly localized to the cytoplasm (Besson *et al.* 2007). These knock-in mice developed more aggressive tumors than the $p27^{Kip1}$-null mice, and by 6 months all the animals showed aggressive pituitary tumors of the anterior lobe. This phenotype seems to be independent of the cell cycle inhibitory activity of $p27^{Kip1}$ and it may be related to the ability of $p27^{Kip1}$ to modulate stem cell function (Besson *et al.* 2007).

Finally, a completely new cell cycle pathway involved in pituitary oncogenesis is represented by PTTG1 (pituitary tumor transforming gene)/securin, an oncogenic molecule first identified in GH4 rat pituitary tumor cells (reviewed in Vlotides *et al.* (2007) and Salehi *et al.* (2008)). PTTG1 is involved in the mitotic checkpoint that prevents abnormal chromosome segregation (see below). In addition, this protein has multiple roles in cell cycle regulation at different stages (Fig. 4). The absence of this gene provokes a decrease in the incidence of pituitary tumors in pRB heterozygous mice, probably by triggering ARF/p53/p21-dependent senescence (Chesnokova *et al.* 2005, 2007). Overexpression of PTTG1 in the pituitary in transgenic mice provokes pituitary hyperplasia and focal microadenomas, and cooperates with pRB heterozygosity in higher incidence of tumors in the AP (Donangelo *et al.* 2006).

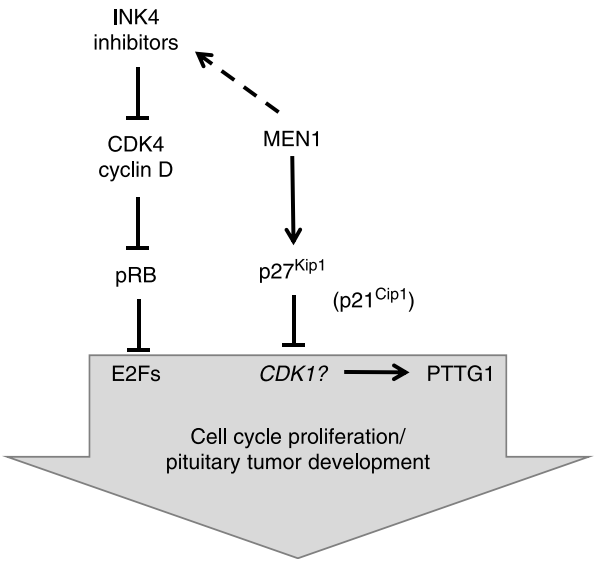

Figure 3 Major oncogenic and tumor suppressor pathways regulating the cell cycle in pituitary tumors.

Deregulation of the cell cycle in human pituitary disease

The experimental analysis of cell cycle control in mouse models predicts that several cell cycle mutations may be present in human pituitary diseases. Pituitary tumors are common intracranial neoplasms that cause significant morbidity through mass effects and/or the inappropriate secretion of pituitary hormones. Pituitary adenomas are common intracranial neoplasms, comprising 10–15% of diagnosed brain tumors (Landis *et al.* 1989). Data from autopsy studies suggest that pituitary adenomas develop in 17–25% of the population (Asa & Ezzat 2002, Ezzat *et al.* 2004). Approximately, 3·5–8·5% of all pituitary tumors are diagnosed prior to the age of 20 years (Keil and Stratakis 2008). About two-thirds of pituitary tumors express and secrete pituitary hormones and produce various endocrine syndromes. Overall, prolactinomas account for about 50% of pituitary adenomas. These adenomas cause hyperprolactinemia and subsequent problems associated to a high level of prolactin in blood (hypoestrogenism or amenorrhea in women or infertility in men). GH-producing adenomas are commonly associated with acromegaly and/or gigantism. ACTH-producing adenomas are associated with Cushing's or Nelson's syndromes (see below). TSH-producing tumors produce thyrotoxicosis, cardiac arrythmias, tremor, and weight loss. The rare gonadotroph adenomas and the major group of non-functionally or non-secreting adenomas result in hypogonadism, visual deficits, and headaches (Asa & Ezzat 2002, Melmed 2003, Ezzat & Asa 2006).

Several genetic and epigenetic alterations have been observed in pituitary tumorigenesis. Some classic oncogenes such as RAS or MYC are implicated in these endocrine tumors. H-RAS mutations (codon 12 (Gly→Val or Arg) or 18 (Ala→Tre)) have been reported only in pituitary carcinomas (Karga *et al.* 1992, Cai *et al.* 1994, Pei *et al.* 1994). c-MYC, on the other hand, is frequently overexpressed in all kind of pituitary tumors in a range between 20 and 50% depending on the type of the tumor (Woloschak *et al.* 1994, Wang *et al.* 1996). Among classic tumor-suppressor genes, p53

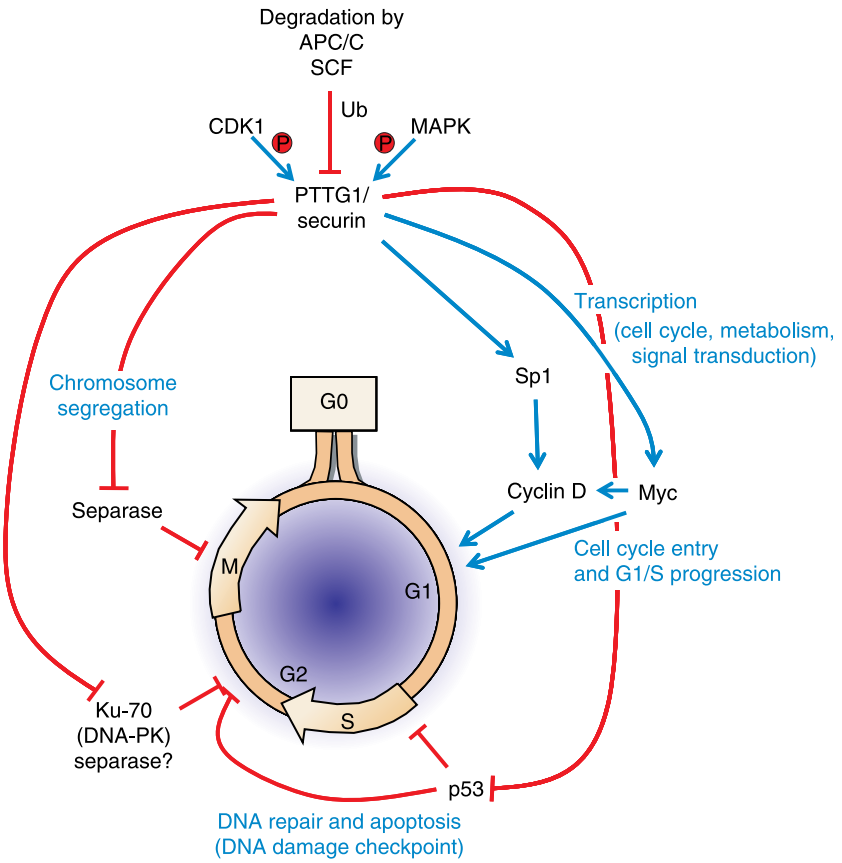

Figure 4 PTTG1/securin functions in the cell cycle (for a comprehensive review see Vlotides *et al.* (2007)).

accumulation (an indication of inactive p53 function) seems to be more relevant in Cushing's adenomas and invasive non-functional tumors than in non-functioning adenomas (Buckley *et al.* 1994, Thapar *et al.* 1996, Clayton *et al.* 1997). In addition to these classic cancer genes, a significant number of genetic or epigenetic alterations in pituitary tumors target several cell cycle regulators as described in the following paragraphs (Table 2). From these data, it has been estimated that more than 80% of pituitary tumors display alterations at least in one of the regulators of the G1/S transition of the cell cycle (Malumbres & Barbacid 2001).

Retinoblastoma protein

Although, early studies did not find loss of pRB alleles (Cryns *et al.* 1993, Zhu *et al.* 1994), later studies found loss of heterozygosity in the human pRB gene (*RB1*) in malignant or highly invasive pituitary tumors (Pei *et al.* 1995; Table 2). Several studies based on immunodetection in tumor sections found abnormal expression of pRB in different pituitary adenomas. In some cases, decreased expression correlates with hypermethylation of the pRB promoter (Simpson *et al.* 2000, Ogino *et al.* 2005) or deletion within the protein-pocket binding domain (Simpson *et al.* 2000).

Cyclins and cyclin-dependent kinase activity

Cyclin D1 and D3 are often overexpressed in pituitary tumors (Jordan *et al.* 2000, Turner *et al.* 2000, Saeger *et al.* 2001, Simpson *et al.* 2001*a*) with some evidence of cyclin D1 allelic imbalance in one fourth of the tumor samples analyzed (Hibberts *et al.* 1999). In general, although cyclin D1 is overexpressed in most pituitary tumor types, this overexpresion is more relevant in non-functional tumors. Cyclin E is also deregulated in human pituitary tumors, with a significant increase in corticotroph neoplasias from patients with Cushing's disease (Jordan *et al.* 2000). Despite the dramatic effect of Cdk4 hyperactivation in mouse models (Table 1), no CDK4 mutations have been identified in human pituitary tumors (Simpson *et al.* 2001*a*, Honda *et al.* 2003, Vax *et al.* 2003).

Table 2 Alteration in cell-cycle regulators in human pituitary tumors

Gene (symbol)	**Cancer-associated alteration** (incidence)	**Tumor type**	**References**
pRB (*RB1*)	LOH (100%)	Highly-invasive or malignant tumors	Pei *et al.* (1995)
	Promoter hypermethylation (60% of non-expressing pRB tumors)	Somatotrophinoma and non-secreting adenomas	Simpson *et al.* (2000)
	Promoter hypermethylation (35%)	Pituitary adenomas	Yoshino *et al.* (2007)
	Promoter hypermethylation (28·6%)	Pituitary adenomas	Ogino *et al.* (2005)
Cyclin D1 (*CCND1*)	Allelic imbalance (25%)	Invasive and non-invasive tumors	Hibberts *et al.* (1999)
	Overexpression (30–50%)	Somatotrophinomas & non-functioning tumors	Simpson *et al.* (2001*a*,*b*)
Cyclin D3 (*CCND3*)	Overexpression (68%)	Pituitary adenomas (all different types)	Saeger *et al.* (2001)
Cyclin E (*CCNE*)	Overexpression (37%)	Cushing's disease adenomas	Jordan *et al.* (2000)
Cyclin A (*CCNA1*)	Overexpression	Pituitary adenomas	Nakabayashi, *et al.* (2001)
$p16^{INK4a}$ (*CDKN2A*)	Promoter hypermethylation (90% of non-expressing p16 tumors)	Different pituitary tumors	Woloschak *et al.* (1997)
	Promoter hypermethylation (59%)	Pituitary adenomas	Yoshino *et al.* (2007)
	Promoter hypermethylation (71·4%)	Pituitary adenomas	Ogino *et al.* (2005)
	Reduced expression levels (62%)	Non-functioning adenomas or	Machiavelli, *et al.* (2008)
	Reduced expression levels (40%)	Macroadenomas (all different types)	Machiavelli *et al.* (2008)
$p15^{INK4b}$ (*CDKN2B*)	Promoter hypermethylation (32%)	Pituitary adenomas	Yoshino *et al.* (2007)
	Promoter hypermethylation (35,7%)	Pituitary adenomas	Ogino *et al.* (2005)
$p18^{INK4c}$ (*CDKN2C*)	Reduced expression levels	ACTH-secreting adenomas	Morris *et al.* (2005)
p27Kip1 (*CDKN1B*)	Reduced expression levels (75% less than 10% cells-expressing in the tumor)	Pituitary adenomas (all different types)	Bamberger *et al.* (1999)
	Reduced expression levels (100%)	Corticotropes & pituitary carcinomas	Lidhar *et al.* (1999)
JAB1 (*COPS5*)	Overexpression (100%)	Pituitary carcinomas	Korbonits *et al.* (2002)
$p21^{CIP1}$ (*CDKN1A*)	Reduced expression levels (71%)	Non-functioning adenomas	Neto, *et al.* (2005)
	Overexpression (77%)	Hormone-producing tumors	Neto *et al.* (2005)
	Overexpression (92%)	GH-producing tumors	Neto *et al.* (2005)
Securin (*PTTG1*)	Overexpression (90% pituitary tumors)	Pituitary adenomas (all different types)	Zhang *et al.* (1999)

INK4 inhibitors

Although point mutations in INK4 inhibitors are not frequent in human pituitary adenomas, the expression of $p16^{INK4a}$ and $p15^{INK4b}$ is often silenced. Silencing of the $p16^{INK4a}$ gene (*CDKN2A*) by hypermethylation was first reported in the late 90s (Woloschak *et al.* 1997). A detailed analysis suggested that *CDKN2A* methylation was confined to particular adenoma subtypes (Simpson *et al.* 1999) and these findings were subsequently confirmed by several other groups concluding that hypermethylation of the *CDKN2A* is the most common epigenetic deregulation in these neoplasias (Morris *et al.* 2005, Ogino *et al.* 2005, Yoshino *et al.* 2007). $p16^{INK4a}$ is able to inhibit cell proliferation in pituitary tumor cells in correlation with a shift in the phosphorylation status of pRB, suggesting the relevance of this CDK inhibitor in the activation of pRB and pituitary tumor suppression (Frost *et al.* 1999).

CIP/KIP inhibitors

Soon after the publication of the phenotype of $p27^{Kip1}$-deficient mice, several studies interrogated the alteration of this inhibitor in human tumors. Early studies detected no $p27^{Kip1}$ mutations in human pituitary tumors (Tanaka *et al.* 1997, Dahia *et al.* 1998). The fact that $p27^{Kip1}$ is haploinsufficient for tumor suppression (Fero *et al.* 1998), however, suggests that decreased expression may be relevant in tumor development. In fact, downregulation of $p27^{Kip1}$ protein expression is commonly observed in pituitary carcinomas and corticotroph adenomas, and recurrent human pituitary adenomas show lower $p27^{Kip1}$ protein levels than non-recurrent adenomas (Bamberger *et al.* 1999, Lidhar *et al.* 1999). $p27^{Kip1}$ mRNA levels are not generally decreased in tumors suggesting increased proteolysis of this cell cycle inhibitor in cancer (Bloom & Pagano 2003). Ubiquitin-mediated degradation of $p27^{Kip1}$ is controlled by SKP2, an F-box protein with diverse oncogenic functions (Frescas & Pagano 2008). Whether SKP2 is the relevant F-box protein for degradation of $p27^{Kip1}$ in pituitary tumors is not yet clear (Musat *et al.* 2002). Degradation of $p27^{Kip1}$ may also be induced by JAB1 (JUN activation domain-binding protein), a transcriptional cofactor for AP-1 (Chamovitz & Segal 2001). In addition to this function, JAB1 is able to translocate phosphorylated $p27^{Kip1}$ to

the cytoplasm for protein degradation by the proteasome. Some pituitary carcinomas display a small but significant increase in JAB1 levels possibly resulting in increased $p27^{Kip1}$ degradation (Korbonits *et al.* 2002). Although, genetic alterations in $p21^{Cip1}$ are not commonly observed, this inhibitor may also be down-regulated through epigenetic modifications in pituitary neoplasias (Yoshino *et al.* 2007, Zhu *et al.* 2008).

Although, the majority of pituitary tumors in humans are spontaneous, in some cases they are part of genetic syndromes predisposing to pituitary and other tumors. These inherited syndromes include multiple endocrine neoplasia (MEN)-1, carney complex, familial isolated pituitary adenomas, and the Cushing's and Nelson's syndromes (Melmed 2003, Beckers & Daly 2007, Keil & Stratakis 2008). The MEN-1 syndrome is characterized by predisposition to pituitary adenomas, parathyroid hyperplasia, and pancreatic endocrine tumors. Pituitary adenomas affect between 25 and 30% of MEN-1 patients (Burgess *et al.* 1998). These patients display germ line mutations in the *MEN1* gene, which increase the susceptibility to all major pituitary adenoma subtypes. MEN1 has been described as a direct regulator of $p27^{Kip1}$ and $p18^{INK4c}$ (Karnik *et al.* 2005, Milne *et al.* 2005), and loss of function of MEN1 results in down-regulation of these two inhibitors with the subsequent deregulation in cell proliferation. In recent mouse models, Men1 mutations cooperate with $p18^{INK4c}$ but not $p27^{Kip1}$ inactivation (Bai *et al.* 2007) suggesting that the MEN1 protein is mostly acting upstream of $p27^{Kip1}$ (Fig. 3). Recently, a mutation in *CDKN1B*, the rat gene encoding $p27^{Kip1}$, has been reported to be associated with a MEN-1-like syndrome in a murine model (Pellegata *et al.* 2006). A germ line nonsense mutation in the human *CDKN1B* gene was also identified in a *MEN1* mutation-negative patient presenting with pituitary and parathyroid tumors. Expanded pedigree analysis showed that the $p27^{Kip1}$ mutation was associated with the development of an MEN-1-like phenotype in multiple generations (Pellegata *et al.* 2006).

PTTG1/securin

PTTG1 was initially identified through a differential display analysis of gene expression in rat pituitary tumor cells (Pei & Melmed 1997). PTTG1, also known as securin, is an inactivating partner of separase, the major effector for chromosome segregation during mitosis (Zou *et al.* 1999). PTTG1 is overexpressed in more than 90% of all type of pituitary tumors (Zhang *et al.* 1999). In addition, this protein is frequently overexpressed in metastatic or genomically instable tumors, suggesting a relevant role for securin in tumor progression (Perez de Castro *et al.* 2007). Securin is regulated by CDK1-mediated phosphorylation (Holt *et al.* 2008) suggesting a link between the control of the cell cycle by CDKs and PTTG1 function (Fig. 3). Despite the frequent deregulation of PTTG1 in pituitary and other tumors, it is not clear yet whether its oncogenic role is mediated by its mitotic functions or the ability of PTTG1 or modulate DNA repair or Sp1-mediated transcription (Vlotides *et al.* 2007; Fig. 4).

Future perspectives and therapeutic implications in pituitary disease

The implication of cell cycle deregulation in pituitary tumorigenesis is well established from experimental data in mouse models (Table 1) and the molecular pathology of human tumors (Table 2). Most cell cycle mutations affect regulators of the G1/S transition in the cell cycle, including the CDK4/pRB pathway and cell cycle inhibitors such as $p27^{Kip1}$ (Malumbres & Barbacid 2001). The role of the pioneer pituitary tumor oncogene PTTG1 is not clear at present, although it may participate in tumor development at different levels. Overall, these mutations provoke a hyperactive cell cycle that ensures unscheduled proliferation and genomic instability in pituitary tumors.

On the other hand, defective cell cycle function also affects pituitary homeostasis. Cdk4 deficient mice are smaller than wild-type littermates and display partial sterility (Rane *et al.* 1999). These phenotypes are linked to hypomorphic pituitaries with a significant decrease in hormone-producing cells. In particular, Cdk4 is required for post-natal proliferation of somato/lactotrophs of the pituitary (Moons *et al.* 2002, Jirawatnotai *et al.* 2004). Some recent results suggest that Cdk4 may also modulate cell proliferation in specific pituitary progenitor cells (Macias *et al.* 2008). Re-expression of Cdk4 in the pituitary rescues the sterility indicating that this defect is secondary to the defects in hormone-expressing cells in the pituitary (Martin *et al.* 2003). However, that re-expression of Cdk4 in the pituitary does not rescue the smaller size of Cdk4-null mice suggesting that dwarfism in these animals is not due to pituitary dysfunction (Martin *et al.* 2003).

Pttg1-deficient mice also display pituitary hypoplasia and decreased proliferation of pancreatic β-cells (Melmed 2003, Vlotides *et al.* 2007). The similarity between Cdk4 and Pttg1 deficiency is striking, although the molecular reasons are unclear. To what extent these cell cycle control pathways contribute to pituitary development and homeostasis is not fully understood yet. However, these experimental results may suggest a relevant relationship between cell cycle regulators and the ability of the pituitary to develop and to respond to physiological stresses. Given the relevance of cell cycle regulators in the correct function of stem cells (Janzen *et al.* 2006, Jablonska *et al.* 2007, Pei *et al.* 2007, Macias

et al. 2008), it is tempting to speculate on the relevance of the cell cycle in pituitary stem cell self-renewal and its implications in pituitary syndromes and tumors.

The observed deregulation of the cell cycle in pituitary disease has important consequences in the treatment of these pathologies. Current treatments in pituitary tumors target neuroendocrine receptors to block hormone–receptor signaling through different pathways (Heaney & Melmed 2004). Currently used drugs include dopamine-receptor agonists and somatostatin analogues. These substances are used to suppress excess hormone secretion and proliferation of pituitary cells, although they also produce several side effects (Heaney & Melmed 2004). The frequent overexpression of cyclins and inactivation of cell cycle inhibitors such as INK4 proteins suggests that CDK hyperactivation is a common theme in pituitary neoplasias. Several small molecular CDK inhibitors are now being evaluated for cancer therapy in many different tumor types (Malumbres *et al.* 2008, Perez de Castro *et al.* 2008). Although, these drugs have not been clinically tested in pituitary tumors, pre-clinical studies suggest that CDK inhibitors may be effective for treating pituitary diseases, at least in individuals with cell cycle mutations that specifically affect this pathway (Sotillo *et al.* 2005). A better knowledge of the specific genetic and epigenetic alterations in human patients will be necessary to select the right combination of current treatments or to propose new therapeutic approaches. Current and future genetic models in the mouse will help us to understand the development of pituitary disorders and to evaluate these therapies before their use in the Clinic.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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3.3.- Un nicho de células GFRa2/Prop1/Stem (GPS) en la hipófisis

La hipófisis adulta es conocida por poseer algunas células productoras de hormonas que regulan procesos vitales fundamentales. Algunas células han sido propuestas como candidatas a células progenitoras/madre. No obstante, poco es conocido acerca de cómo la hipófisis se regenera durante la vida adulta y de su regulación homeostática durante determinados cambios fisiológicos específicos, como la pubertad o el embarazo, o en condiciones patológicas como el desarrollo tumoral. En este trabajo, hemos identificado en roedores y humanos un nicho de células no endocrinas caracterizado por la expresión de GFRa2, un correceptor tipo Ret para Neurturina. Estas células también expresan las proteínas b-catenina y e-caderina orientadas de una manera específica, sugiriendo una polaridad planar en la organización del nicho. Además, las células del nicho expresan el factor de transcripción específico de células de la hipófisis Prop1, así como los marcadores de células progenitoras/madre Sox2, Sox9 y Oct4. Las células positivas para GFRa2 fueron capaces de formar esferoides en cultivo que no producían hormonas. Estos esferoides eran capaces de diferenciar a células productoras de hormonas o neuronas bajo estímulos específicos. *In vivo*, las células GPS presentan bajos niveles de proliferación tras el nacimiento, siendo capaces de retener BrdU y manteniendo los telómeros largos, propiedades características de las células progenitoras/madre in vivo. El porcentaje de GPS presentes en la hipófisis se ve alterado en animales deficientes para Cdk4, un modelo de hipófisis subdesarrollado. En consecuencia, las células GPS tendrían una relevancia funcional en la expansión fisiológica de la hipófisis durante la vida así como protectora de la enfermedad.

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Contribución del doctorando al trabajo

Victor Quereda realizó el trabajo en ratones, excepto la cuantificación de la longitud de los telómeros. Realizó el análisis de las inmunohistoquímicas de muestras humanas. Colaboró en el diseño de los experimentos y en la escritura del artículo.

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A GRFa2/Prop1/Stem (GPS) Cell Niche in the Pituitary

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Abstract

Background: The adult endocrine pituitary is known to host several hormone-producing cells regulating major physiological processes during life. Some candidates to progenitor/stem cells have been proposed. However, not much is known about pituitary cell renewal throughout life and its homeostatic regulation during specific physiological changes, such as puberty or pregnancy, or in pathological conditions such as tumor development.

Principal Findings: We have identified in rodents and humans a niche of non-endocrine cells characterized by the expression of GFRa2, a Ret co-receptor for Neurturin. These cells also express b-Catenin and E-cadherin in an oriented manner suggesting a planar polarity organization for the niche. In addition, cells in the niche uniquely express the pituitary-specific transcription factor Prop1, as well as known progenitor/stem markers such as Sox2, Sox9 and Oct4. Half of these GPS (GFRa2/Prop1/Stem) cells express S-100 whereas surrounding elongated cells in contact with GPS cells express Vimentin. GFRa2+-cells form non-endocrine spheroids in culture. These spheroids can be differentiated to hormone-producing cells or neurons outlining the neuroectoderm potential of these progenitors. In vivo, GPSs cells display slow proliferation after birth, retain BrdU label and show long telomeres in its nuclei, indicating progenitor/stem cell properties in vivo.

Significance: Our results suggest the presence in the adult pituitary of a specific niche of cells characterized by the expression of GFRa2, the pituitary-specific protein Prop1 and stem cell markers. These GPS cells are able to produce different hormone-producing and neuron-like cells and they may therefore contribute to postnatal pituitary homeostasis. Indeed, the relative abundance of GPS numbers is altered in Cdk4-deficient mice, a model of hypopituitarism induced by the lack of this cyclin-dependent kinase. Thus, GPS cells may display functional relevance in the physiological expansion of the pituitary gland throughout life as well as protection from pituitary disease.

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Introduction

The pituitary gland is a central endocrine organ that regulates basic physiological functions such as growth, stress response, reproduction, lactation and metabolic homeostasis. The adenopituitary (AP) hosts several endocrine cell types secreting hormones that regulate the function of other organs and endocrine glands throughout life. Thus, somatotrophs, lactotrophs and thyrotrophs secrete growth hormone (GH), prolactin (PRL), and thyroid-stimulating hormone (TSH) respectively; corticotrophs secrete adrenocorticotropic hormone (ACTH) and gonadotrophs secrete luteinizing hormone (LH) and/or follicle-stimulating hormone (FSH). In addition, some non-hormonal folliculostellate cells have been described whose function is not well understood [1–3]. All these cells in the AP arise during development from a common ectodermal primordium known as the Rathke's pouch [4]. However, not much is known on pituitary cell renewal throughout life and its homeostatic regulation during specific physiological changes such as puberty or pregnancy or in pathological conditions such as tumor development. To explain these changes, both cell proliferation of the individual differentiated secretory cells and asymmetric proliferation followed by terminal differentiation of adult stem cells have been proposed [5,6].

Although the identity of adult pituitary stem cells is not well established, several stem/progenitor cell types have been previously proposed to maintain pituitary homeostasis and generate endocrine cells. A side population (SP) that efficiently excludes the Hoechst 33342 vital dye has been shown to segregate with sphere-forming cells in the pituitary [7]. In addition, pituitary colony-forming cells (PCFCs) that display notable clonogenic potential

have also been isolated [8]. However, the only common marker studied for these cells was Sca1 and their position in the pituitary was not well understood [7,9]. Recently, the presence of Sox2+/Sox9− of the mouse pituitary has been described and proposed to mark stem cells, localized both as an epithelial layer but also heavily intermingled with the differentiated cells [10], while more differentiated progenitors or transit-amplifying cells would become Sox2+/Sox9+. Genetic approaches using transgenic mice expressing GFP under the Nestin promoter identified a population of Nestin+ cells that in vitro behaves as progenitors; however, these cells would only contribute post-puberally to cell-renewal in the adult pituitary [11].

In this manuscript we describe a niche of putative stem cells that express the Glial cell line-derived neurotrophic factor (GDNF) receptor alpha 2 (GFRa2). GFRa2 belongs to a family of receptors (GFRa1-4) that modulate signaling pathways initiated by their ligands, GDNF, Neurturin (NTN), Artemin (ART) and Persephin (PSP). These proteins function as co-receptors of the tyrosine kinase Ret [12–14]. GFRa2 functions as an specific NTN receptor as demonstrated in vivo by the almost identical phenotype of mice deficient in either NTN or GFRa2 [15,16]. In some tissues such as testis and ovary, GFRa1 and 2 receptors are expressed in putative germ-line stem cells [17–19]. In the pituitary, somatotrophs (GH) are the only secretory cells expressing Ret and GFRa1 either in rat [20] or in humans [21]. We report here that GFRa2 is expressed in a niche of non-hormonal putative stem/progenitor cells in the pituitary. GFRa2-positive (GFRa2+) cells are organized in a single-cell layer around the cleft originated from the Rathke's pouch. These niche cells display a clear expression of the pituitary specific homeobox protein Prophet of Pit1 (Prop1), a transcription factor required for pituitary development and mutated in pituitary disease [22–26]. In addition, these niche cells also express well-established stem cell markers such as Oct4, Sox2, Sox9 and we will refer to them as GPS (**G**FRa2+, **P**rop1+, **S**tem) cells.

Results

GFRa2 expression is mostly restricted to a polarized niche in the pituitary

GFRa2 is expressed in the rat pituitary at similar levels to testis (Figure S1A) or ovary (data not shown), two other endocrine glands where GFRa1 and 2 receptors had been previously described [17–19]. In the murine pituitary, GFRa2 expression is restricted to a distinct subset of non-endocrine cells lined to a single-cell layer in the marginal zone (MZ) around the cleft between the intermediate lobe (IL) and the AP (Figure 1). The MZ had been proposed to harbor stem/progenitor cells originated from the Rathke's pouch from which the endocrine cells could be produced but still no clear proof has been found [5,9]. This layer of cells opposed to the cleft originates, like the AP, from the Rathke's pouch formed from the oral ectoderm during embryonic development. A very limited number of isolated GRFa2+ cells are distributed throughout the AP (Figure 1A). In total, GRFa2+ cells accounts for about 0.9% cells of the adult mouse pituitary (Figure S1B). GFRa2+ cells do not express any pituitary hormone (Figure 1B) but shows a significant expression of epithelial markers such as Cytokeratins and E-cadherin (Figure 1C–D and Table 1). These GFRa2+ cells also display a clear expression of b-Catenin (Figure 1C –rat- and D –mouse-), whose labeling is rarely positive in other cells of this endocrine gland (Figure S1-C). The GFRa2+ niche in the MZ seems to have a Planar Polarity organization. Thus, the anti-GFRa2 antibody stains a very thin line in the coronal axis of the MZ cells. However, GFRa2 stains broadly on MZ cells in the axial axis. Similar polarization is found with b-Catenin where each cell appears as a U-shaped line in coronal sections versus complete rings in axial sections (Figure 1C and Videos S1 and S2). Interestingly, the GFRa2 (membrane) and b-Catenin (cytoplasm) signals are perpendicular (see Axial 400× and 1000× sections), suggesting that the GFRa2 cell niche is formed of cylindrical cells with Planar Polarity coordination, a specific coordination of an epithelial layer of cells to behave with a physiological direction (recently reviewed in [27].

GFRa2+ cells express pituitary specific factors and stem cell markers in murine and human pituitaries

A variety of stem/progenitor cell markers is also expressed in the niche of GFRa2+ cells (Table 1). Among them, the recently described [10] Sox 2 and Sox9 transcription factors (Figure 2A –mouse- and 2B-rat-) that co-stain with GFRa2/b-Catenin. These cells also display a clear signal for Oct4 (Figure 2C).

GFRa2 cells does not express the pituitary specific transcription factor Pit1 (Figure S2-A) but display a clear and specific signal for Prop1 (Figure 2D). Prop1 is a transcription factor known for its exclusive expression in pituitary development. Mutations in the Prop1 gene cause hypopituitarism due to Combined Pituitary Hormone Deficiency (CPHD) in humans [23] and the Ames dwarfism in mice [22,25].

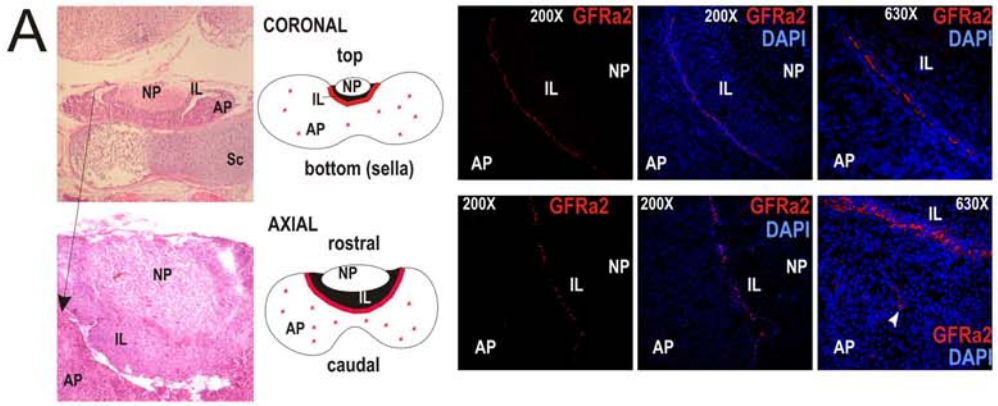
Based on the fact that GFRa2+ cells express a pituitary specific factor, Prop1, with clear physiological relevance (see Discussion), and *bona-fide* stem cell markers such as Sox and Oct4 proteins, we call them GPS (**G**FRa2+, **P**rop1+, **S**tem). GFRa2+ cells also express SSEA4 (Figure 2E), a glycolipid marker of embryonic stem cells However, the niche of cells is negative for other stem markers such as Nanog –expressed in the IL (Figure 2E) and S2B-, Sox4, Isl-1 or Pax6 (Figure S2 and Table 1). Whereas the other co-receptor, GFRa1, is not expressed in the GPS cells, they are positive for the Ret receptor although with less intensity than somatotrophs (Figure 2E and S2-C).

The calcium-binding protein S-100 (a marker of folliculostellate cells [28–30]) is present in about 50% of GPS cells, in addition to many scattered and elongated cells in the AP, MZ and IL (Figure 2F). Another marker of folliculostellate cells, Vimentin [31,32], delineates the GPS niche (Figure 2G). However, double immunofluorescence with beta-catenin does not show the expected co-localization. Vimentin+ cells appear as a line of elongated cells posterior to the GPSs just before the IL both in rat and mouse pituitaries (Figure 2G, H). Finally, Nestin, a marker of some folliculostellate cells [33] is expressed in long and thin processes through the AP, IL and NP similar to neurons or to folliculostellate cells, but it does not correlate with GFRa2 staining (Figure 2I).

A similar niche of GPS cells, expressing GFRa2, Oct4, Sox2, Sox9 and is also present in the MZ of the human pituitary around the so called Rathke's remnant cysts (Figure 3A–B). The human pituitary also expresses Prop1 (Figure 3C). The niche of human GPS is also partially positive for S-100 but negative for Vimentin, which is expressed by elongated cells in the same area just in contact with the GPS (Figure 3D). On the other hand, the GFRa2-specific ligand NTN is exclusively expressed in groups throughout the AP and not at the niche either in human or in rat pituitary (Figure 3E–F and Figure S2-F). This finding, together with the planar polarity found in the GPS niche, suggests a functional asymmetric signaling in which the GFRa2/RET/NTN pathway may be implicated.

GFRa2+ cells form embryonic-like spheroids capable to differentiate in hormone-producing cells

To address the differentiation potential of the GPS niche, we isolated GRFa2+ individual cells and maintained them as a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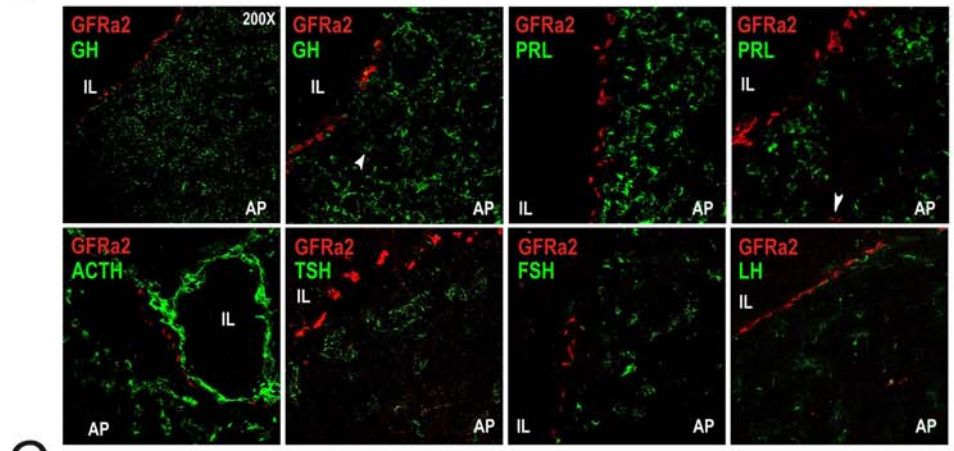
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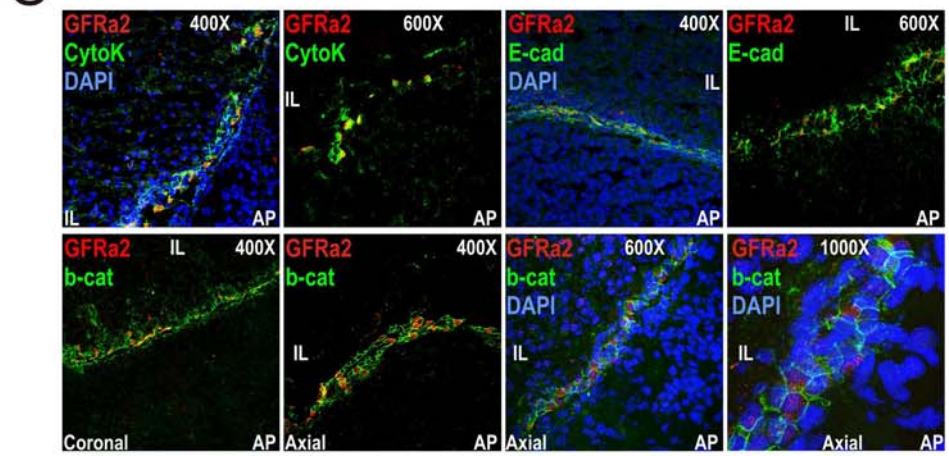
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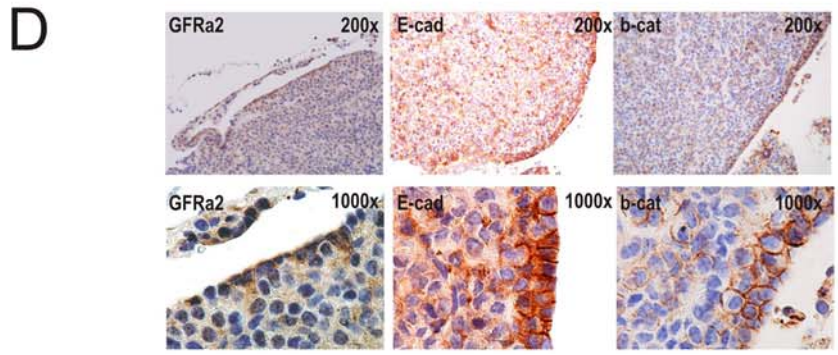
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Figure 1. GFRa2+-expressing cells form a line of epithelial non-secretory cells in the adult pituitary of rats and mice. A) Coronal and axial sections stained with Hematoxylin and Eosin (H&E) to show pituitary location under the hypothalamus and on top of the sphenoid sella turcica (Sc) and the disposition of the three pituitary lobes: adenopituitary (AP), intermediate lobe (IL) and neuropituitary (NP) where end-terminals of hypothalamic axons release ADH and Oxytocin. In the rat pituitary, GFRa2+ cells (red) arrange in a precise line in the frontier between the AP and the IL. Very few less intense cells are found dispersed through the AP (arrowhead). **B)** GFRa2+ cells [either lined or scattered (arrowhead)] do not express any pituitary hormone. **C)** GFRa2+ cells are epithelial cells with enhanced expression of Cytokeratins, E-cadherin and beta-Catenin. Coronal versus Axial sections demonstrates the orientation of the GFRa2 cells within the niche. In the coronal axis, GFRa2 or b-Catenin appear respectively as a thin line or a U-shaped green staining; in the axial axis GFRa2 appears as a broad surface while b-Catenin shows a ring shaped staining in a perpendicular orientation. **D)** Localization of GFRa2 cells and co-localization with E-cadherin and b-Catenin in mouse pituitaries.
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suspension culture in a serum-free conditioned medium (SpherM). 2500 cells either GFRa2+ or GFRa2−negative were seeded in a 35-cm-diameter dish (around 800 cells/ml). After seven days, while the GFRa-negative dishes presented a few clumps of cells (12 clumps/dish, 4–8 cells/clump), the GFRa2+ cells formed spheroid structures either compact or hollow with an empty cavity surrounded by small cells (>139 spheroids/dish, around 40 cells/spheroid; average of more than 50 experiments). Some of the spheroids contain cilia and display active movements (Figure 4A and Videos S3, S4, S5 and S6). One of the roles of planar polarity in embryogenesis is to induce oriented cilia during morphogenetic migration, and functional defects in these cilia cause embryonic abnormalities [34]. In humans, GPS are located around the reminiscent Rathke's cleft (see Figure 3), which has been described to present cilia [35,36]. A benign non-neoplastic disease called Rathke's cleft cyst (RCC) is in fact characterized by a MZ cyst pathognomonically surrounded by ciliated cells [37]. These cysts are thought to originate from remnants of the Rathke's pouch and, in fact, their localization is similar to what shown in Figure 3 for human GPS cells.

Table 1. Markers expressed by GFRa2+ cells.

Marker	% of GFRa2+ cells containing the marker*
Hormones	
GH	0%
ACTH	0%
PRL	0%
TSH	0%
FSH	0%
LH	0%
Epithelial markers and Wnt pathway	
Multi-Cytokeratin	90%
E-cadherin	86%
b-Catenin	91%
GFRa2 pathway	
Ret	78%
GFRa1	0%
NTN	0%
Stem cell markers	
SSEA4	100%
Prop1	99%
Oct4	94%
Sox2	91%
Sox9	96%
Sox4	0%
Nestin	0%
Nanog	0%
Isl-1	0%
Pax6	0%
Other Markers	
S-100	43%
Vimentin	<3%
Proliferation markers	
Ki67 in adult GPS	0%
Ki67 at 10 days	13%

*Percentages are calculated counting cells from (confocal) microscopy pictures (magnification higher than 400×). For each combination of markers, between 125 and 200 cells where counted from at least three independent pituitaries.
doi:10.1371/journal.pone.0004815.t001

The spheroids actively divide up-to 50 cells (Figure 4B). Opposite, the GFRa2 negative fraction maintained in parallel remains as isolate cells with a few 4–8 cell clumps and does not grow (BrdU-negative, Figure 4B, right, white bars). To demonstrate that the spheroids are clonal, i.e. originate from a single cell, we diluted the GFRa2+-cell suspension to 18 cells/ml and seeded 1 ml/well in the first column of a 24-well dish. Dilutions 1:2 were performed in the next columns up to 0.5 cells/well. After five days spheroids were carefully looked out and all of them photographed to count approximately the number of cells per spheroid (Figure S3-A, a representative experiment with quadruplicates is shown). All the spheroids found were multicellular (ranging from 25 to >100 cells/spheroid). The number of individual spheroids per well were proportional to the number of individual GPS cells seeded per well. Even diluting at 0,5 cell/ml we found near one multicellular spheroid per well.

The spheroids maintain GRFa2 expression and display positive labeling for Oct-4, Prop1, E-cadherin, and b-Catenin but are hormone-negative (Figure 4C–D and Fig S3-B). The GFRa2 ligand, Neurturin (50 ng/ml) has a trophic effect in the spheroid number when culturing in a diluted (0.5×) SpherM (Figure S3-C) indicating the GPS dependence of a functional RET/GFRa2/NTN pathway. If the pituitaries are dispersed with trypsin (instead of Collagenase) no single spheroid grows from the few GFRa2+ purified cells. This may be explained by possible deleterious effects of trypsin in the extracellular domain of GFRa2. Similarly, if the spheroids are dispersed with trypsin, they are able to make secondary spheroids albeit the number of secondary spheroids obtained was 1/3 of the number of GPSs seeded.

In the presence of gelatin and conditioned-media from MEFs (50% MEFM), these GFRa2/Prop+ cells attach to the well and grow slowly as a scattered culture (Figure S3-D). However, after the second passage, differentiated structures as "cord-like" structures, colonies expressing red pigmentation or other kind of defined-cells appear under the microscope intermingled with the scattered GFRa2+ cells. We don't know at present if the GPSs have multipotent capacity. When the GPS cells are cultured on top of mitomycin-treated MEFs in the presence of the characteristic medium for Stem cells (StemM), they grow as undifferentiated

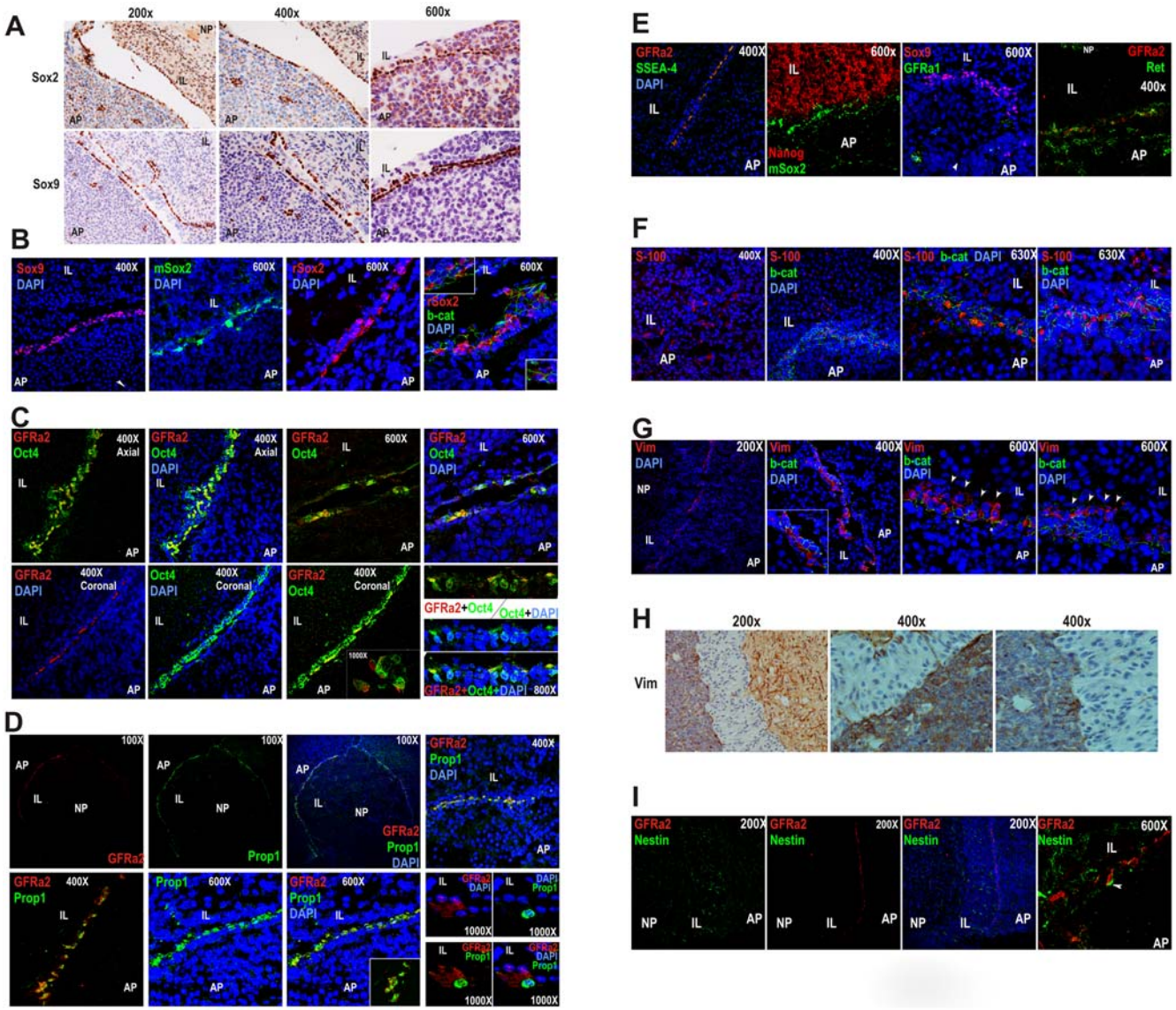

Figure 2. The GPS Niche: GFRa2 cells express Prop1 and stem cell markers while neighbor cells express Vimentin. A) Detection of Sox2 and Sox9 in the mouse and the rat pituitary (mSox2: mouse monoclonal and rSox2: rabbit polyclonal anti-Sox2 antibodies). **(B)**. Sox2 signal co-localizes with b-Catenin. **C)** In rat pituitary, Oct4 is also expressed in the same line of cells, and co-localizes with GFRa2. **D)** Co-localization between GFRa2+ and Prop1 in the marginal zone between the AP and IL. Notice the nuclei positive for Prop1 surrounded by the GFRa2 membrane staining. **E)** GFRa2 cells co-localize with SSEA4, a glycolipid characteristic of Stem cells, but not with Nanog, which is restricted to the IL. GFRa2 cells do not express GFRa1 (which is however observed in somatotrophs) but weakly express the Ret receptor (Fig. S2). **F)** S-100 is expressed by the folliculostelate cells of the IL and AP of rat pituitary, but is also concentrated in around half of the b-Catenin/GFRa2 cells. **G)** Vimentin, a mesenchymal stem cell marker, is also expressed in the same niche as the GFRa2 cells but not in the same cells. Towards the IL, a parallel line of elongated cells (arrows) just beyond the b-Catenin/GFRa2 cells (asterisks) can be observed; fixation provokes sometimes the separation of both lines of cells (right panel). A similar Vimentin staining is seen in mouse pituitary **(H). I)** Although Nestin is expressed in the three portions of the pituitary, GFRa2 cells are negative for Nestin expression. Thin structures similar to axons apparently coming from the Nestin+ neuropituitary contact the GFRa2 cells (arrowhead).

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colonies and display cilia (Figure S3-C and Video S7). In the presence of MEFM supplemented with LIF (ESGRO), these cells do not attach to the gelatin-coated dish but grow as floating spheres. We have been able to maintain these cultured GPS cells either as attached/floating colonies or spheres at least up to the 7th passage and still continue (Figure S3-E).

We next asked whether GFRa2+ spheroids maintain the capability to differentiate to endocrine cells. Single spheroids were isolated by pipetting under the phase-contrast microscope and placed on Collagen Type IV coated wells, the collagen characteristic of basal membranes from epithelial layers. We next induced attachment with serum for one day, followed by incubation in medium containing a specific combination of supplements (DifM 1–4, see Methods). The spheroid got attached during the first 24 hours of culture in presence of serum. From that moment on, the cells start to attach to the dish and the spheroid progressively disappears. Some of the cells migrate very far away from the point where the spheroid attaches. If the spheroid was big many cells appear on the dish; if the spheroid was small less reduced numbers appear. That means that although we

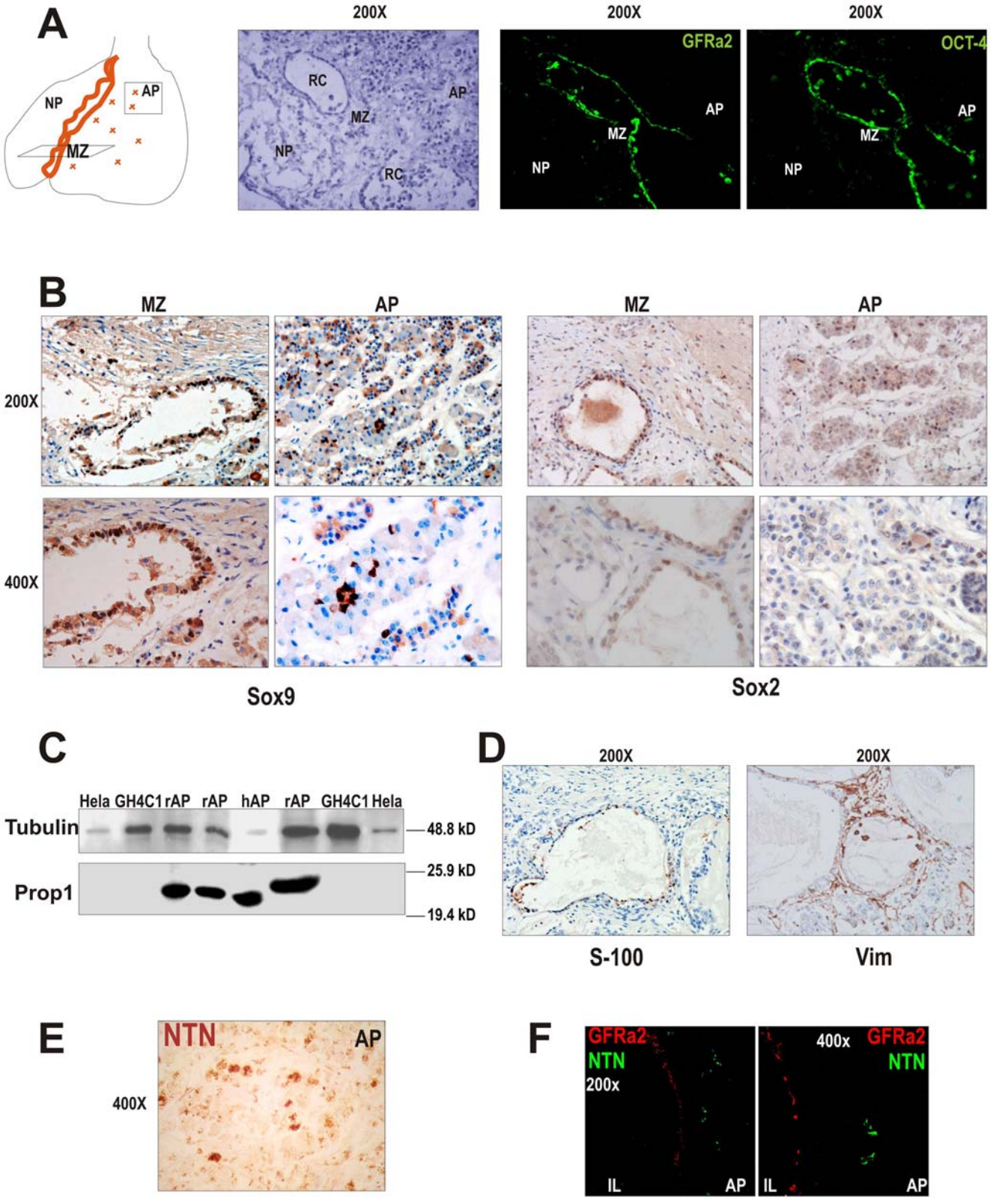

Figure 3. The human marginal zone (MZ) of the pituitary contains a similar niche of GPS cells. A) Cartoon representing the anatomy of the human pituitary with the anterior AP and a posterior NP; the boundary is called MZ and contains dilated structures usually called Rathke's remnant's Cysts (RC). Cells lining the RC express GFRa2 and Oct4. **B)** These cells also express Sox9 and Sox2. The human pituitary also contains small groups of Sox9+ or Sox2+ cells within the AP. **C)** Western blot detection of the pituitary specific factor Prop1 protein in rat (rAP) and human (hAP) pituitary, but not expressed in HeLa cells or a somatotroph pituitary cell line (GH4C1). **D)** S-100 is expressed in around half of the human GFRa2 cells lining the RC, similarly to what observed in the rat pituitary. Similarly, Vimentin+ elongated cells surrounded the GFRa2 epithelium (right panel). **E)** The GFRa2 ligand NTN is expressed in the human and rat **(F)** pituitary, and localizes exclusively at the AP.
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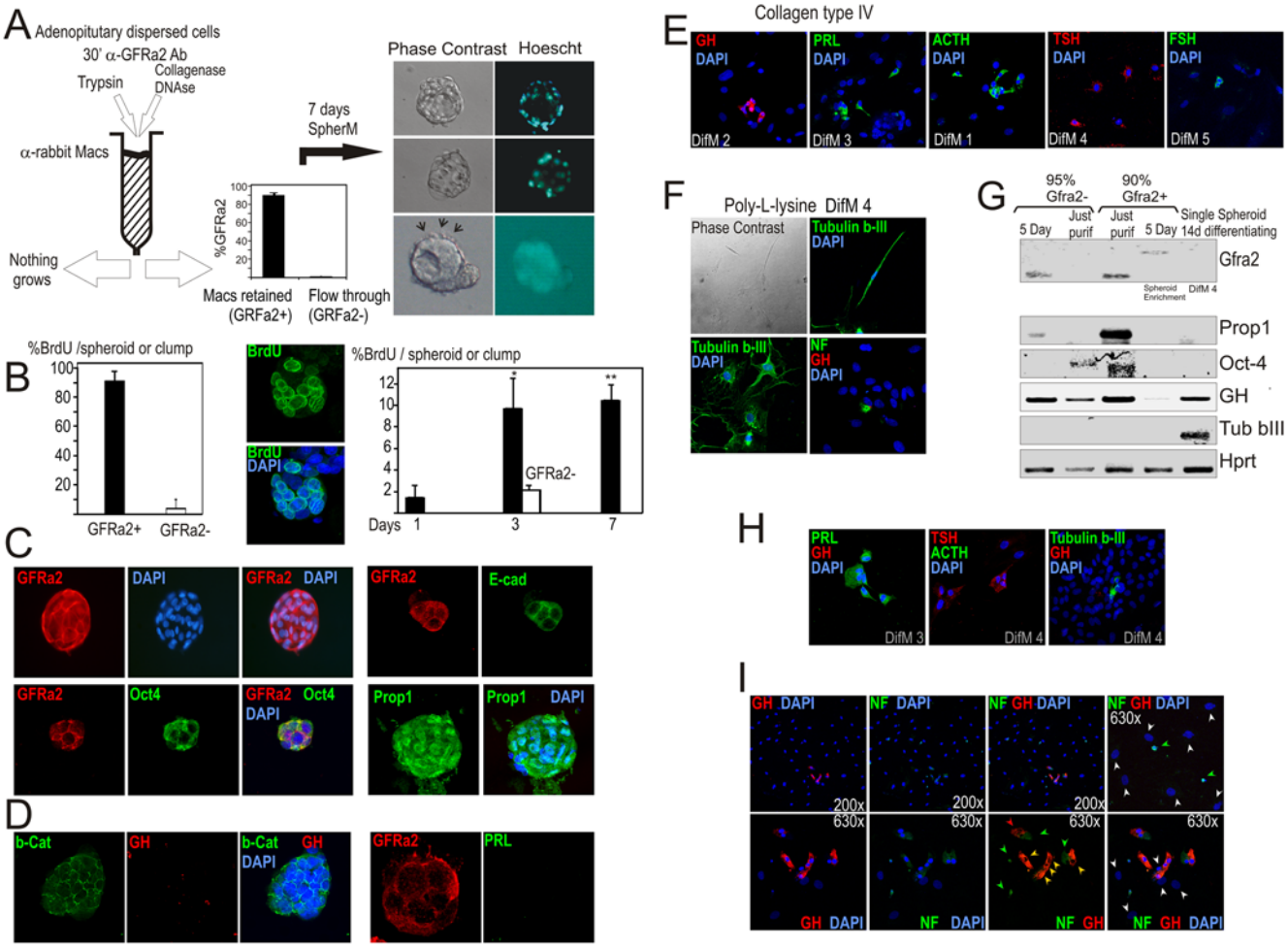

Figure 4. Purified GFRa2+ cells form embryonic-like spheroids that differentiate into different ectodermic cell types. A) Rat AP single-cell dispersions are prepared by treatment with Collagenase. The two fractions obtained, a GFRa2+ purified fraction (90% positivity for GFRa2 by immunofluorescence) and the Flow through GFRa2- (95% negativity for GFRa2), are then kept in SpherM. After 7 days, spheroids formed by small cells are observed in the GFRa2+ fraction. Some of they contain a hollow cavity while others are compact. A bunch of moving cilia is frequently observed in one pole of these spheroids (arrows; see videos in Supplementary Information). AP cell dispersion with trypsin does not result in viable spheroids as GFRa are extracellular receptors sensitive to trypsin treatment. **B)** Proliferation in the GFRa2+ and GFRa2- fractions after 5 days in the presence of BrdU. Center: A 7-day-old spheroid incubated with BrdU only for the last 12 hours before fixation. Right: BrdU uptake within growing spheroids incubated with BrdU during the last 12 hours before fixation. **C)** The spheroids are clonal (see Figure S3-A) and express GFRa2, Oct4, Prop1 or E-cadherin. **D)** These structures express b-Catenin but not hormones such as GH, PRL (and S3-B) or ACTH (data not shown). **E–I)** A single spheroid was transferred under the microscope to a collagen/poly-lysine-coated well and attached to the matrix with serum for 24 h. Spheroid structure disappear and cells spread through the well. Cells differentiate depending on the culture conditions into **E)** different pituitary secretory types (intermediate nuclei) or **F)** neurons (small or big nuclei) showing Tubulin beta III-+ cells+ or NF+ cells. **G)** mRNA expression of GFRa2/Prop1/Oct4 in the GFRa2+ purified fraction. The GFRa2+ fraction still have some contaminating secretory cells expressing GH. Five days later (spheroids) RNA expression of GFRa2 shifted (alternative splicing) while Prop/Oct4 were negative (even if the proteins were present). 14 days after differentiation of a single hand-picked spheroid, expression of either secretory (GH) or neuronal (Tubulin b-III) differentiation markers is detected. **H)** Double immunofluorescence in differentiated spheroids showed that differentiation is most frequently driven towards either secretory or neural phenotype. **I)** However, in some wells double GH/NF+ cells (orange arrows) together with single GH+ (red arrows) or NF+ (green arrows) or negative (white arrows) cells coexisted (Table 2).

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cannot exclude the possibility of proliferation after the induction of attachment/differentiation we have the repeated impression from the many experiments that the differentiated cells do not proliferate. Using this approach, we were able to differentiate these spheroids into GH-, PRL-, TSH-, ACTH-, or FSH-producing cells (Figure 4E). We did not observed any cell positive for GFRa2 or Prop1 after differentiation (Table 2). When spheroids are seeded on top of Poly-L-lysine with DifM4, Tubulin-beta-III positive cells are observed. Tubulin-beta III is characteristic of neurons and in fact some of these cells present bipolar appearance (Figure 4F), suggesting an ectodermal stem cell potential for pituitary GRFa2+ cells. Similar results were obtained using the characteristic neuronal intermediate filament protein Neurofilament (NF).

To evaluate RNA expression throughout the differentiation process, we performed RT-PCR analysis in the GFRa2+ fraction (90% pure), the GFRa2-negative fraction (95% pure), in both fractions 5 days after culture in SpherM (when there is a spheroid-enrichment in the GFRa2+ fraction), and in the cells obtained after differentiation of a single spheroid in DifM4 (Figure 4G). GPS stem markers are strongly expressed in the GFRa2+ fraction and absent in GFRa2-negative cells. In parallel, GH expression is

Table 2. Summary of all the differentiation experiments with the 5 differentiation media (DifM1-5)*.

	GH		PRL		bTSH		ACTH		bFSH		Tub b III		NeuroF	
	n°+	nuclei	n°+	nuclei	n°+	nuclei	n°+	nuclei	n°+	nuclei	n°+	nuclei	n°+	nuclei
DifM1	n.t.		n.t.		0	10			n.t.		n.t.		n.t.	
							9	38						
							10	18						
DifM2	**1(1)**	25							**5 (1)**	25	n.t.		n.t.	
	1(1)	52					**12(1)**	52						
			0	17										
			0	17										
					6 (0)	29	*0 (0)*	29						
					7 (0)	9	*0 (0)*	9						
	8(0)	27							*0 (0)*	27				
	3(2)	58							**2 (2)**	58				
DifM3	*0 (0)*	17	*6 (0)*	17			n.t.		n.t.		n.t.		n.t.	
					0	15								
			6(**a**)	51										
	0 (**b**)	18												
			2(**a**)	3										
	0	13												
DifM4 (Polylysine)							n.t.		n.t.		5	15		
					26(0)	**27**					**1(0)**	27		
					7 (0)	28					*0 (0)*	28		
	6(5)	93											**10(5)**	93
	3 (0)	9											*0 (0)*	9
	7 (0)	37									*0 (0)*	37		
	0 (0)	117									*2 (0)*	117		
	0 (0)	50											*1 (0)*	50
	0 (0)	25									*24 (0)*	25		
			0	18										
DifM5	n.t.		n.t.		n.t.		n.t.		2	31	n.t.		n.t.	

*A single isolated spheroid was induced to attach to the gelatine-coated (DifM1,2,3 and 5) or poylysine-coated (DifM4) well with serum for one day, and induced to differentiate during 15 days in the presence of any of the DifM 1 to 5. Wells were fixed and immunofluorescence performed. In some wells double immunofluorescence was performed (aligned in the same row): those wells where more than one cell type co-existed are written in bold and the number of cells double positives for both markers is shown in brackets; those wells where one single type of cell was detected are in italics. In some of the wells (underlined), co-immunofluorescence for GFRa2 (**a**) or Prop (**b**) was performed, always with a negative result.

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still present after purification, probably due to the abundance of somatotrophs in the pituitary (10% contaminating GFRa2-cells). After culturing in SpherM medium, expression of GPS markers decay although the proteins are still present in the spheroids, and GFRa2 band shifts to a different spliced band. No differentiation markers, Tubulin-beta III, or a very weak band of GH can be detected. It seems that the GPS cells in the spheroid structures start to change its characteristics but need later inputs from different ligands (present in the various DifM) to get differentiated; some of these differences are downregulation of Oct4, Prop1 mRNA expression or alternative splicing of GFRa2 mRNA. In the GFRa2- fraction (95% GFRa2-) with days positivity for GFRa2 and Prop1 mRNA appear demonstrating the self-renewal capacity of the few remaining GFRa2+ cells.

When the spheroids are induced to differentiate, no GPS markers are detected but GH and Tubulin-beta III are expressed de novo. In our hands, differentiation protocols are quite specific since lactotrophs (PRL) are only obtained with DifM 3, whereas GH was never detected in this medium (Table 2). When two markers were simultaneously analyzed in the same well, the majority of differentiated cells are positive for one marker and there was only one type of differentiated cell (blue on Table 2, Figure 4F and H-left). However on occasion two types of differentiated cells co-existed on the same well (green on Table 2, Figure 4I, lower magnification), and even there were some cells positive for two hormones or for GH and NF (pink on Table 2, Figure 4I, higher magnification). In general, positivity for hormones is linked to a nucleus of intermediate size; positivity for NF correlates with a nucleus of small size; and positivity for Tubulin-beta III is frequently observed in cell with a large nucleus.

Slow proliferation and long telomeres in the GPS niche of adult animals

In vivo the stem cells are slow cycling cells that retain the nuclear DNA label of infancy into adult age as demonstrated for mouse skin, mammary gland, endometrium and liver [38–41], or

for rat pancreas and kidney [42,43]. The GPS niche already exists in newborn-rat pituitaries (Figure 5A). GRFa2+ cells actively divide during early postnatal development but loose proliferative potential with age, as detected by Ki67 staining of the GPS niche (Figure 5A and B). Also in adult mouse pituitaries, cell division is scarce and rarely observed in GPS cells (Figure 5C), thus suggesting that GPSs were slow cycling cells in vivo. In parallel, the expression levels of stem cell markers in the AP decrease during postnatal development to adulthood in an inverse correlation with the production of hormones or the Pit1 transcription factor (Figure 5D), implying a division of stem cells to increase pituitary mass. To analyze the frequency of replication in the putative stem cell niche, we used the BrdU retaining technique. In rats, GRFa2+ cells specifically retained BrdU even 60 days after an injection when newborns whereas this signal was lost in most of the other cells in the pituitary (Figure 5E). These results suggest that the niche of GFRa2 cells replicates slowly after birth, a property shared by most progenitor/stem cells.

Slow replication is linked to long telomeres and these two features are a hallmark of stemness [44–46]. Mature cells have usually undergone many divisions and telomere length gradually decreases with each cell cycle due to incomplete replication of telomeric DNA. We have used a novel technique, "telomapping", to quantify the length of telomeres in situ based upon the specific in-situ hybridization of a fluorescent telomeric DNA probe on paraffin sections. The longest telomeres in the pituitary specifically mark the marginal zone within the IL/AP boundary where GPS cells are located (Figure 5G). Progressive rows of cells towards the AP or NP present less intense signals (orange) while the mature secretory cells in AP have the faintest signal (green) corresponding to short telomeres. These results suggest that progenitor cells in the pituitary are located to the MZ where GPS localize. On the other hand, most cells in the AP display short telomeres suggesting an abundant component of mature cells that have undergone many cell divisions.

Altered cell cycle regulation of GPS cells in genetically-modified mouse models with hypo- or hyperplastic pituitaries

Proliferation in the progenitor/stem cell niches depends on Cdk4 activity, being carefully downregulated within the niche and increasing when the progenitor cell enters in the so-called transit-amplifying state to become differentiated [47]. Cdk4-deficient mice in which Cdk4 has been inactivated by the insertion of a neomycin-resistant (neo) cassette [Cdk4(n/n) mice] [48] display hypoplastic pituitaries with a dramatic decrease of all hormone-secretory cells in the AP during postnatal life (Figure 6A–B, center and Figure S4). These Cdk4-null pituitaries display normal Ki67 staining during embryonic development but a decreased proliferation after birth (data not shown), similarly to that we have previously observed in Cdk4-null endocrine cells in the pancreas [49]. Yet, the ratio between GPS cells and total number of cells in the pituitary was not only maintained but enlarged in these animals suggesting normal production of these cells during embryonic development but abnormal differentiation into hormone-producing cells. Thus, whereas wild-type mice contain about 0.9% of GPS cells (see Figure S1), these cells display a relative 3-fold enrichment in Cdk4(n/n) mice (Figure 6C). Moreover Cdk4-null pituitaries display long telomeres throughout the AP (Figure 6D), suggesting a reduced number of cell cycles in these small anterior pituitaries. Hypoplastic Cdk4 KO pituitaries do not exhibit an enriched "niche" in absolute terms. The niche is relatively enriched considering the reduced number of endocrine-producing cells. The interpretation is in keeping with the concept that most defects in stem cell proliferation result in a defect in differentiated cells without affecting the stem compartment. A similar situation has been found previously in the hematopoietic compartment of Cdk4/6 deficient embryos [50].

Interestingly, these three phenotypes (reduced pituitary size and cellularity, relative increase in GPS cells and long telomeres in the AP) are rescued when Cdk4 is re-expressed [Cdk4(R/R) mice] by expressing Cre recombinase and removing the neo cassette (Figure 6A–D, right panels), in parallel with the recovery of normal pituitary function (Figure S4). These results suggest that Cdk4 participates in the control of postnatal proliferation and/or differentiation of GPS cells.

Discussion

The existence of a primordial cell in the pituitary was proposed more than ten years ago when exceptional human pituitary adenomas were observed to concomitantly express Pit1-dependent hormones (GH, PRL and TSH) plus ACTH and gonadotrophic hormones [51–53]. More recently, the presence of stem cells in the pituitary has been suggested in dispersed cultures isolated by citometry as a Side Population (SP cells) of mouse pituitary cells positive for Sca1, Nestin, Nanog and Oct4, but negative for Prop1 [7]. Additional progenitor cells have been also proposed as a colony-forming population of Sca1+ and angiotensin-converting enzyme (ACE)-+ cells [9]. Some of the later were in fact located to the MZ of the pituitary. The MZ had been proposed to harbor stem/progenitor cells originated from the Rathke's pouch from which the endocrine cells could be produced [1,9]. Recently, Sox2+/Sox9− cells have been found in the mouse MZ but also strongly dispersed throughout the pituitary intermingled with secretory cells [10]. A population of Nestin+ cells has been traced after birth in the pituitary in vivo [11]. Nestin+ cells were found in the three parts of the pituitary, and only a small population of the adult secretory AP cells was originated from these Nestin+ cells postpuberally, more than 2.5 months after birth. At present, it is unclear whether the growth of the pituitary after birth or maintenance of the adult population of secretory cells requires a single or several types of progenitor/stem cells.

We have characterized a specific cell population in the MZ of the rodent –rat and mice– and human pituitary, initially identified by the expression of the GFRa2 receptor. These cells exhibit unique features, i.e. not present in other pituitary cell-types, such as the presence of GFRa2 receptors, the expression of the pituitary specific transcription factor Prop1, and the presence of additional stem cell markers such as Sox2, Sox9, SSEA4 and Oct4. The presence of these markers, long telomeres, and the in vitro potential of GPS cells to differentiate in all AP endocrine cells make them strong candidates for the maintenance of differentiated cells on the pituitary. GPS cells are Nanog/Nestin-negative but similar Oct-4+ Multipotent Adult Progenitor Cells (MAPCs) have been reported to be negative for other embryonic stem cell markers such as Nanog or Sca1 [54]. SSEA-4 is an embryonic stem cell marker in humans but it is not present in mouse embryonic stem cell lines, which are instead positive for SSEA-1. There is not known in much detail what markers are present on the surface cells of the early rat embryo stages, if they would be lacto-series of glycolipids (SSEA-1) as in mouse or globo-series of glycolipids (SSEA-3 and SSEA-4) as in humans. Rat embryonic stem cells recently obtained expressed SSEA-4, SSEA-3 and SSEA-1 on top of Oct4 or Nanog [55]. In agreement with these results, it was known that a small percentage of rat Dorsal Root Ganglia (DRG) "small" precursors were positive for SSEA-4 [56]. The DRG is one of the niches where neural crest progenitors/stem

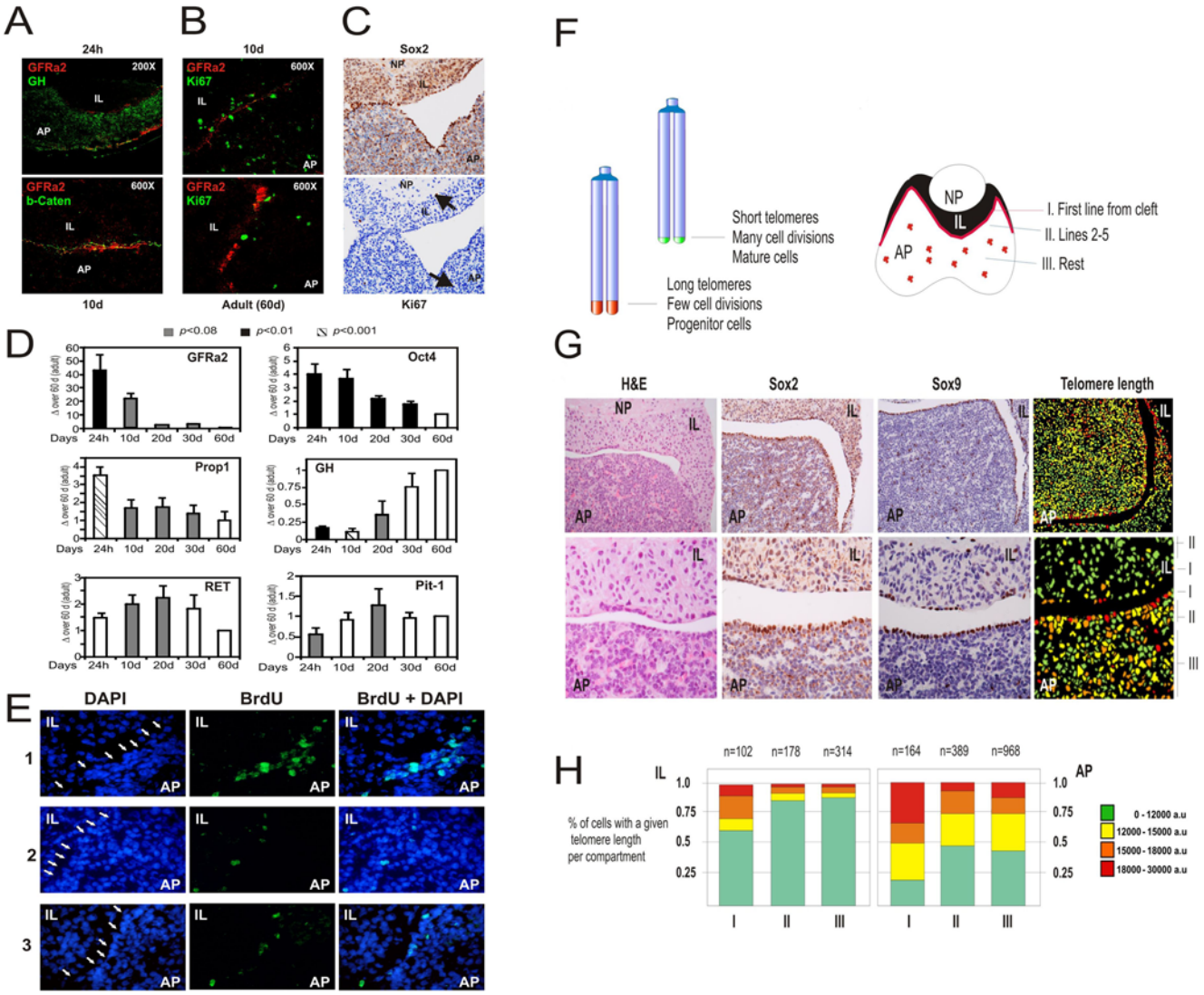

Figure 5. GFRa2+ niche is present at birth and maintained through adulthood with reduced proliferation and long telomeres. A) Newborn (24 h) and 10-days (10 d) old rat pituitaries present a GFRa2+/b-Catenin+ but GH- niche similar to that adult pituitaries (60 to 90-days old). **B)** 10 d pituitaries display abundant cell proliferation in the GPS niche, as seen by Ki67 staining, opposite to the adult rat (60 d) or mouse organs **(C)**. **D)** Expression of GFRa2, Oct4, Prop1, GH, Ret and Pit1 in the AP of newborn and 10-, 20-, 30- and 60-day-old rats as detected by qRT-PCR. GPS progenitor markers (GFRa2, Oct4, Prop1) decrease with age while somatotroph markers (Ret, Pit-1) peak around puberty, day 10 to 20, or increase with grow to adulthood (GH). **E)** BrdU retaining in the GPS niche (arrows). Adult-pituitary nuclei within the niche retain BrdU injected in the rats as newborns. Three different animals (1–3) are depicted in the figure. **F)** Telomapping analysis of normal mouse pituitaries demonstrates a thin line of very long-telomere containing nuclei exactly in the first row of cells at the IL/AP boundary (regions I) matching the GPS niche. The following rows of cells towards the AP or the IL/NP present a shortening of the telomeres while the bulk of secretory cells have short telomeres characteristic of differentiated cells. **G)** Normal pituitaries were stained with H&E, Sox2 or Sox9 showing the GPS cells in the AP/IL boundaries (AP region I and IL region I) and some scattered groups through the AP (mostly in region III of the AP). Telomapping analysis as quantified in **H)** indicates that the region I of AP contains most long-telomere cells. This percentage progressively decreases in region II and III, where scattered GPS cells with long telomeres are found. In the IL, the only cells with long telomeres are also located in region I of the IL. AP, adenopituitary; IL, intermediate lobe; NP, neuropituitary.

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cells reside ([57] and many previous references therein). The mouse vs. human difference present into embryonic stem cells, changes in adult stem cells. Recently, it has been demonstrated that hematopoietic stem cells are positive for SSEA-4 (and Sca1 positive but c-Kit/CD45/Flk-1/and SSEA-1 negative) both in mouse and in human and, in fact, SSEA-4 has been proposed to better purify HSCs from the bone marrow [58].

Adult stem cells divide infrequently and reside in protected microenvironments or niches [59] with a low rate of telomere erosion throughout their life-time. These niches can be either acellular or contain other cell types that give support to the stem cell niche [60]. The presence of Vimentin+ cells near the GPS cells in both rodent and human pituitary suggests the presence of a cellular stem cell niche on the pituitary. GPS cells also express E-cadherin and b-Catenin in a polarized manner (coronal vs. axial) surrounded by Vimentin+ cells, suggesting a putative relation with the known function of the Wnt/b-Catenin pathway together with E-cadherin to retain the stem cells within the niche confines [60]. Future studies

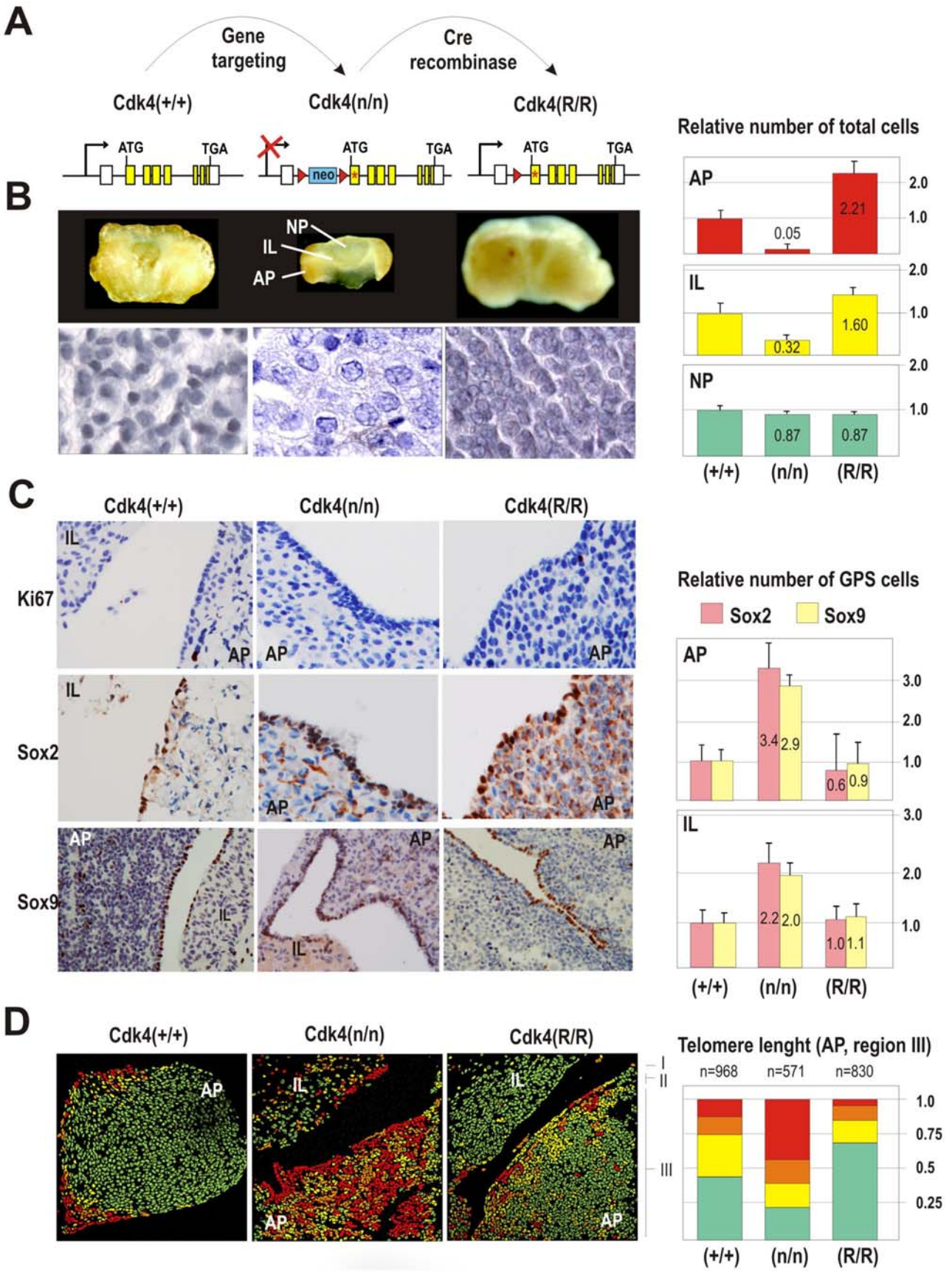
A
Gene targeting
Cre recombinase
Cdk4(+/+)
Cdk4(n/n)
Cdk4(R/R)
ATG
TGA
neo
B
NP
IL
AP
Relative number of total cells
AP
2.21
0.05
IL
1.60
0.32
NP
0.87
0.87
(+/+)
(n/n)
(R/R)
C
Cdk4(+/+)
Cdk4(n/n)
Cdk4(R/R)
Ki67
Sox2
Sox9
Relative number of GPS cells
Sox2
Sox9
3.4
2.9
0.6
0.9
2.2
2.0
1.0
1.1
D
Telomere lenght (AP, region III)
n=968
n=571
n=830
I
II
III

Figure 6. Increased relative abundance of GPS progenitors and decreased formation of endocrine cells in Cdk4–null mice. A) Alleles used in the analysis of GPS cells in a hypopituitarism model. The Cdk4-null allele Cdk4(n) is obtained by insertion of a neo-resistance cassette in Cdk4 intron 1. This mutation is rescued by expressing, through Cre recombination, a $Cdk4^{R24C}$ mutant allele that encodes a hyperactive Cdk4 [48]. **B)** Cdk4-null mice display a hypoplastic pituitary much smaller than the wild type due to low cellularity and smaller size. These differences mostly affect the AP, containing around 5% cells of the wild-type pituitaries by 2-month age. This phenotype is rescued in the Cdk4(R/R) which displays a bigger pituitary with a 2-fold increase in the AP by 2-months. **C)** Genetic ablation of Cdk4 does not affect the structure of the stem cell niche. Moreover, the relative abundance of GPS cells is enlarged presenting more cell layers and 3–3.5-fold more GPS cells in the AP and 2-fold in the IL. **D)** The overall length of telomeres is significantly increased in the Cdk4-deficient AP distal region III. About 48% of these cells display long telomeres whereas this number is about 12% in wild-type or $Cdk4^{R24C}$-rescued AP. AP, adenopituitary; IL, intermediate lobe; NP, neuropituitary.
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assessing a role of the canonical Wnt pathway on this niche are clearly merited. Perpendicular staining of GFRa2 versus b-Catenin within the GPS niche reminds of planar polarity, a specific coordination of an epithelial layer of cells to behave with a physiological direction (recently reviewed in [27,61]. It is therefore not casual that isolated GPS cells in culture form moving embryonic-like spheroids and present specialized cilia. One of the roles of planar polarity in embryogenesis is indeed to induce oriented cilia during morphogenetic migration to prevent embryonic abnormalities [34]. The planar polarity in the GPS niche also suggests a functional asymmetric signaling in which both the GFRa2 and Wnt pathways may be implicated. Thus, the Ret/GFRa2 pathway may help to indicate the cells in the niche the correct side to migrate and/or to proliferate. The expression of the ligand NTN in discrete cells through the AP but not in the niche cells adds an interesting suggestion of luring the GPS cells out of the niche through a guiding gradient. This situation is reminiscent of the niche present in the seminiferous tubules of the testes where Ret and GFRa1/GFRa2, along their ligands GDNF and NTN, play an important role in the interplay between multipotency versus differentiation of the germ stem cells [18,19,62]. Similarly, in the pituitary, the NTN/GFRa2 axis may modulate stem/progenitor physiology whereas Ret/GFRa1 system controls somatotroph differentiation and fate (death versus survival) through Pit1 regulation as we have described previously [63].

Some additional genes expressed by the GPS niche, such as Sox proteins or Prop1, have important roles in pituitary physiology and disease. Mutations in the Sox2 gene cause pituitary hypoplasia associated with hypogonadotrophic hypogonadism and eye, ear and encephalic abnormalities [64–67]. Ames dwarf mice and Prop1 null mice have a normal pituitary volume at birth but the organ does not grow nor differentiate in postnatal life. Similar phenotype have the patients affected by Combined Pituitary Hormone Deficiency (CPHD) [23], a hypopituitarism caused by mutations in Prop1 [68] where there pituitary undergo progressive hormone loss suggesting a depletion of progenitors. Patients with CPHD display a general loss in all types of secretory cells, but affecting more those that are required throughout growth and puberty (GH, LH/FSH) and metabolism (TSH). Although initially it may be not present, delayed ACTH deficiency also appears [69]. Some patients present with hyperplasia of the pituitary while others display hypoplastic pituitaries; moreover, it is currently accepted that enlargement of the pituitary precedes the hypoplasia [69,70]. Ames dwarf mice have a slightly different phenotype, with the predominant failure in the Pit1-dependent secretory types (GH, PRL, TSH) and apparently not deficiency in gonadotrophs/corticotrophs [22,71]. Since this in a spontaneous mutation we cannot be sure of the genetic background. However, Prop1-deficient mice have display a phenotype similar to the human CPHD, including gonadotroph deficiency [25]. Both Ames dwarf and Prop1-deficient mice have a normal (or only slightly decreased) pituitary at birth, suggesting a defect in adult homeostasis. Moreover, Prop1 transgenic mice have a delay in puberty [72]. Prop1 in the pituitary embryonic progenitor cells of the Rathke's pouch is considered to play a role in the migration process of the progenitor cells out of the marginal zone [26,73]. Our data demonstrate that Prop1 expression in the adult pituitary is restricted to the GPS niche. It is tempting to speculate a role for Prop1 in protecting the stem cells and correctly guide them through asymmetric division/differentiation when needed. A detailed study of the niche in these animal models will be performed.

Interestingly, the pituitary deficiency induced by Prop1 mutations is reminiscent of the hypopituitarism induced by inactivation of the cell cycle regulator Cdk4 ([74] and Figures 6 and S4). GPS cells are present in this model although accompanied by an overall decrease in endocrine producing cells. These AP cells display longer telomeres suggesting a defective number of cell divisions from their progenitor cells. Interestingly, GFRa2-deficient mice display a significant failure to thrive after weaning although the involvement of pituitary function in this phenotype has not been addressed [16]. Also similarly to Cdk4, persistent Prop1 expression in the mouse delays endocrine differentiation and enhances tumor susceptibility [72] (see below).

The initial Rathke's pouch, as well as the encephalic neural tube, comes from the anterior ectoderm. GFRa2+ spheroids are able to differentiate to secretory pituitary cells but also towards neuron-like phenotypes when driven appropriately with a specific differentiation medium. A similar induction of neuronal phenotypes from epithelial stem cells of the inner ear has been demonstrated ([75]. Similarly, pituitary secretory cells can be obtained from neuronal fetal progenitors [76] and many human pituitary adenomas present with neural metaplasia [77]. However, the GPS are able to remain undifferentiated when grown in conditioned-medium from MEFs; in this conditions, however, part of the cells differentiate spontaneously with passages. GPS remain undifferentiated and form colonies when grown directly on top of MEFs or when grown in the presence of ESGRO (LIF), a feature shared by all stem cells described.

All together, our results suggest that GPS cells may have relevant contributions to postnatal pituitary homeostasis. These cells a likely to form a functional niche of adult precursor cells with functional relevance in the physiological expansion of the pituitary gland throughout life as well as protection from pituitary disease.

Materials and Methods

For a detailed list of methods and antibodies and dilutions see Supplementary Methods S1 and Table S1, S2 and S3.

Human and murine samples

Rats were obtained from the Central Animal House of the USC, a registered animal facility that maintains the animals under welfare and ethical conditions complying with the 86/609/CEE, RD223/88, and OM 13/10/89 laws. The project had the approval of the Ethical Committee of the USC. Rat pituitaries were obtained from adult (200–250 gr., 60 days) male/female Sprague-Dowley rats. To study expression during postnatal development newborns, 10, 20, 30 and 60 days old male rat pituitaries were compared.

Human pituitary samples were selected from the archives of the Department of Pathology, Hospital Universitario Virgen del Rocío (Sevilla, Spain). Informed consent was required from patients according to the policies of the Ethical Committee of the Hospital.

Generation and characteristics of the Cdk4-deficient mice has been previously described [48,78–80]. The Cre strain used was CMV-Cre [48]. Mice were maintained in a mixed 129/Sv×C57BL/6J background following the institutional guidelines at the Spanish National Cancer Research Center (CNIO) and the protocol approved by the Committee of Bioethics and Animal Care of the Comunidad de Madrid. The animals were observed in a daily basis and sick mice were euthanized humanely in accordance with the Guidelines for Humane End Points for Animals used in biomedical research.

Immunodetection

For immunofluorescence, rat pituitaries were oriented and immersed in an OCT-filled plastic cryomold (Sakura) and frozen inside a glass beaker filled with isopentane previously immersed in liquid N2; frozen cryomolds were maintained at −80°C until sectioned in 10 microns cryosections. The sections were fixed with 0.1% Paraformaldehyde for 10 minutes (GFRa2, Prop-1, Oct-4, Citokeratins, E-cadherin, SSEA-4, rabbit Sox2, Nanog, Nestin, ACTH, PRL, FSH, LH) or with -20°C methanol for 5 minutes (GFRa2, Prop-1, Oct-4, b-Catenin, rabbit anti-Sox2 (rSox2), Nanog, Ret, GFRa1, GH, TSH) or 0.5% paraformaldehyde for 20 minutes (GFRa2, GH, Ki-67); for mouse anti-Sox2 (mSox2) and Sox9 the pituitaries were fixed in 4% paraformaldehyde overnight before freezing and sectioning. Alternatively, cryosections were fixed in 4% paraformaldehyde for at least 10 minutes. Primary antibodies were applied overnight in PBS, thoroughly washed in PBS followed by 1 hour incubation with secondary antibodies, washing and mounted using GelMount (Biomeda). Guinea pig polyclonal antibody anti- Prop1 was made in house against the carboxy-terminal domain of mouse Prop-1. cDNA encoding amino acids 151 to 223 were cloned downstream of either a GST or a His-tag vector. Fusion proteins were expressed in BL21 E. coli and partially purified over glutathione agarose (Sigma) or Ni2+-NTA-agarose (Qiagen). Initial immunizations were performed with GST-Prop-1 fusion protein and the final boosts were performed with the His-Prop-1 fusion proteins. It has been already demonstrated that this antibody recognizes Prop1 transcription factor in mouse E12.5 [81]. Double immunofluorescences were performed in consecutive days; to prevent secondary antibody backgrounds, the order was dependent on the species of the primary antibody: first day goat, guinea pig or rabbit, second day rabbit or mouse respectively. Negative (using PBS instead of primary antibody) and preadsorption (competing with cold peptide/protein) controls were routinely run in parallel (see Supplementary Methods S1). Nuclei were counterstained with 20 μg/ml DAPI (Sigma). A TCS-SP2-DMRE Confocal Microscope with Ar, He/Ne 543 and He/Ne 633 Lasers (Leica) and LCS software was used to analyze the results.

For immunocytochemistry and telomapping, mouse or human pituitaries were fixed in 10% buffered formalin at 4°C, dehydrated through graded alcohols and xylene, and embedded in paraffin. Prior to embedding, pituitaries were oriented in order to obtain specific sagittal or coronal 5 microM sections. Prior to IHC, paraffin-embedded slides were de-paraffinized, re-hydrated, immersed in 10 mM citrate solution and epitopes retrieved by three high-power, 5 min microwave pulses. Slides were washed in water, blocked in 1:10 dilution of normal goat serum (Vector Labs) and incubated with primary antibodies. Slides were then incubated with secondary biotinylated antibodies followed by signal development with an immunoperoxidase reagent (ABC-HRP, Vector Labs) and DAB (Sigma). Sections were lightly counterstained with hematoxylin and analyzed by light microscopy.

For immunoblotting, tissues o cells were lysed as previously described [63,82].

Isolation and culture of GFRa2+ cells

A detailed protocol is provided as Supplementary Information. Briefly, freshly isolated cell suspensions were prepared from male rat or mouse pituitaries using magnetic activated cell sorting (MACS; Miltenyi) or a fluorescence-activated cell sorter (FACS; FACSAria, Becton-Dickinson). The experiments with spheroids shown in the Figures 4 and S3 were performed with rat cells, although many have been reproduced in mouse cells (data not shown).

GFRa2+ purified cells were cultured in un-coated wells in the presence of SpherM. After 5–7 days spheroids were either video-recorded or fixed for further immunofluorescence or induced to differentiate. For the BrdU-uptake experiments, 10 microM BrdU (Sigma) was added from the beginning, but a toxic effect was seen with longer treatments than 5 days; to evaluate the % of cell division in spheroids of different days BrdU was added for the last 12 hours of incubation before fixation. To differentiate each spheroid was carefully picked with a P1000 pipet under the microscope and placed in poly-L-lysine or Collagen type IV treated Cultureslides (BD) in 10% FCS-SpherM. The following day, the medium was replaced during 14 days by any of the differentiated media DifM 1–4. Immunofluorescence of spheroids was performed pipetting them on top of 8 microM inserts (Millipore) and fixing them with 70% Ethanol at room temperature during 30 minutes, plus 4 M HCL during 20 minutes (BrdU labeling) or with −20°C Methanol for 5 minutes for the other antibodies before proceeding as above. Differentiated cells were fixed in Methanol (hormones) or in 4% paraformaldehyde for 20 minutes (hormones, Tubulin-beta III, NF).

BrdU retaining technique

Three days old rats were injected subcutaneously with 50 μg/g BrdU (Sigma) in 0.9% NaCl twice/day during 3.5 days. 60 days later, animals were sacrificed and pituitaries frozen as above. Cryosections were fixed in −20°C Methanol for 10 minutes, washed and incubated in 4 M HCl for 20 minutes. After washing, immunofluorescence with anti-BrdU (BD) was performed as above.

Confocal quantitative telomere FISH (Telomapping)

For telomapping, paraffin-embedded tissue sections were hybridized with a PNA-tel Cy3-labelled probe and telomere length was determined as described [46]. DAPI, Cy3 signals were acquired simultaneously into separate channels using a confocal ultraspectral microscope (Leica TCS-SP2-A-OBS-UV) using a PL APO 20×/0.70 PH 2 as lens with Leica LCS software and maximum projections from image stacks (10 sections at steps 1.0 microM) were generated for image quantification. The DAPI image was used to define the nuclear area and the Cy3 image to quantify of telomere fluorescence. The binary DAPI mask was applied to the matching Cy3 to obtain a combined image with telomere fluorescence information for each nucleus. Cy3 fluorescence intensity (telomere fluorescence) was measured as "average gray values" (total gray value/nuclei area) units (arbitrary units of fluorescence). These "average telomere fluorescence" values always represent the average Cy3 pixel intensity for the total nuclear area, and not the average value of individual telomere spot

intensities, therefore ruling out that differences in nuclear size may influence telomere length measurements.

Supporting Information

Figure S1 Expression of GFRa2 and b-Catenin in the adenopituitary A) GFRa2 mRNA expression in the rat adenopituitary (AP) is comparable with the testes, a gland well known for its GFR alpha expression. B) GFRa2 stains about 0.9% of all AP cells detected by flow cytometry after specific binding of anti-GFRa2 antibody. The enzyme dispersed suspension of mouse-adenopituitary cells were sequentially incubated with anti-GFRa2 antibody followed by FITC-anti-rabbit antibody (see Supplementary Tables S1 and S2). The cell suspension was analyzed by cytometry; in red the analyzed FITC+ population and in blue the sorted population presenting the strongest FITC signal. In the negative control (only Ig, secondary antibody) this population was less than <0.1%, while in the GFRa2+ samples accounted for around 0.9% of the total cell suspension. The GFRa2+ population is composed of homogeneous small cells as seen by the low level of the sorted population on the FSC in comparison with the non-FITC population or with the faint FITC+ within the control. C) Low magnifications of a whole section of a rat pituitary (DAPI) and the b-Catenin enrichment at the niche between AP and IL. The only pituitaries small enough to picture like this were from 10-days old rats. AP, adenopituitary; IL, intermediate lobe; NP, neuropituitary.
Found at: doi:10.1371/journal.pone.0004815.s001 (0.92 MB PDF)

Figure S2 The GPS niche is weakly positive for RET but does not express Pit-1, Nanog, GFRa1, Nestin or Sox4. A) The GPS niche is negative for Pit1, a pituitary transcription factor expressed by somatotrophs (GH), lactotrophs and thyrotrophs, as is negative for GH. B) The GPS niche is also negative for Nanog. Nanog staining is only observed in the IL and does not overlap with b-Catenin at the niche. C) The Ret tyrosine-kinase receptor stains specific cells in the AP (mostly somatotrophs, [1,2], and it is also expressed in neurons of the NP. It also weakly stains the GFRa2+ niche; however the GPS cells are negative for GFRa1. D) The Nestin+cells of the pituitary are dispersed through the IL and the AP [3], but do not coincide with the GPS. E) Sox4 is expressed in the mouse AP but it is not a marker of the GPS niche. F) Western blot of GFRa2 and Neurturin (NTN) in rat and human adenopituitary. Hela cells are a human positive control for GFRa2. PRL has a slight interspecies difference in MW.
Found at: doi:10.1371/journal.pone.0004815.s002 (1.71 MB PDF)

Figure S3 Differentiation and proliferation properties of GFRa2-purified cells in vitro. A) The spheroids are clonal: A representative experiment is shown where GFRa2+ cells were diluted in SpherM to 18 cells/ml and seeded into the first column of a 24-well dish. Further dilutions 1:2 were performed in the following wells. Five days later all the spheroids per well were counted (white numbers in the middle of the wells) and photographed to be able to appreciate an approximate number of cells/spheroid. In those wells where more than 4 spheroids were found, a picture of four of them is shown. B) GFRa2 spheroids express Prop1 and thin lines of b-Catenin and are negative for PRL. C) Neurturin (NTN), the GFRa2 ligand, functions as a physiological promoter of spheroid formation when cells are cultured under sub-optimal conditions (0.5×: medium diluted by half) of SpherM culture media. D) Three ways of culturing MACS purified GFRa2+ cells render different phenotypes: a) In uncoated dishes with SpherM, GFRa2+ grow as spheroids as described; b) cultured on gelatin-coated dishes using 50% of conditioned medium from MEFs (MEFM), they attach to the surface and grow as GFRa2+/Prop1+ scattered cells. However, with passages some differentiated groups of cells forming cord-like structures or red-pigmented colonies appear and the scattered GPS cell number is less; c) when cultured directly on top of mitomycin-treated MEFs (as frequently used for embryonic stem cells), GFRa2+ cells form colonies that present cilia in the apical pole (Supplementary Video 6). E) Adding Esgro to the MEFM (MEFM+E), the cells did not attach to the gelatin-coated surface, but grew slowly but steadily as compact spheres. They were passaged every 25 days. We show here four independent cultures five days after passage. As expected, GPS cells cultured on top of MEF carried on with passages forming colonies (black arrows), although some isolated differentiated cells appeared.
Found at: doi:10.1371/journal.pone.0004815.s003 (1.32 MB PDF)

Figure S4 Cdk4 null mouse but not Cdk4(R/R) has hypopituitarism A) Sagittal microphotographs of pituitaries from Cdk4(+/+), Cdk4(n/n) and Cdk4(R/R) 2-month-old mice. B) The total number of hormone-producing cells is decreased in young (2–4 months-old) Cdk4-deficient mouse pituitaries and they have smaller pituitaries (panel A and Figure 6). However, the relative percentage of hormone-producing cells is not grossly altered in Cdk4-deficient mice, suggesting an overall deficiency in the production of all these cells from these progenitors. C) Adult female mice present a physiological increase in lactotroph cells in comparison with males that is maintained in the Cdk4-null mice, in spite of having a much less number of total lactotrophs. Cdk4(R/R) mice recover normal amount of lactotrophs. In the left, serum prolactin levels in the animals were analyzed by immunoassay. D) Representative images of hormone-producing cells in Cdk4(n/n) mice.
Found at: doi:10.1371/journal.pone.0004815.s004 (0.34 MB PDF)

Video S1 Three dimensional reconstruction of the rat AP niche using b-Catenin staining (green).
Found at: doi:10.1371/journal.pone.0004815.s005 (1.81 MB AVI)

Video S2 Three dimensional reconstruction of the rat AP niche using all channels together, DAPI (Nuclei) blue, GFRa2 (red membrane staining), and b-Catenin (green).
Found at: doi:10.1371/journal.pone.0004815.s006 (1.78 MB MPG)

Video S3 Spheroid with beating cilia in one pole.
Found at: doi:10.1371/journal.pone.0004815.s007 (1.05 MB AVI)

Video S4 Hollow spheroid moving against another.
Found at: doi:10.1371/journal.pone.0004815.s008 (5.24 MB AVI)

Video S5 Hollow spheroid moving fast through the culture dish.
Found at: doi:10.1371/journal.pone.0004815.s009 (3.55 MB AVI)

Video S6 Compact spheroid with cilia.
Found at: doi:10.1371/journal.pone.0004815.s010 (2.32 MB AVI)

Video S7 Colony of GFRa2+ cells grown on top of mitomycin-treated MEFs for two weeks, with cilia beating on the surface.
Found at: doi:10.1371/journal.pone.0004815.s011 (2.74 MB AVI)

Methods S1
Found at: doi:10.1371/journal.pone.0004815.s012 (8.22 MB DOC)

Table S1 List of Antibodies and dilutions. Primary Antibodies

Found at: doi:10.1371/journal.pone.0004815.s013 (0.06 MB DOC)

Table S2 List of secondary antibodies and related reagents.
Found at: doi:10.1371/journal.pone.0004815.s014 (0.04 MB DOC)

Table S3 Oligonucleotides used to analyze gene expression by RT-PCR.
Found at: doi:10.1371/journal.pone.0004815.s015 (0.03 MB DOC)

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Author Contributions

Conceived and designed the experiments: MGL VQ MAJ AKR MAB CD MM CVA. Performed the experiments: MGL VQ IF CS EDR. Analyzed the data: MGL VQ IF CS MAJ MM CVA. Contributed reagents/materials/analysis tools: AKR MAB MM CVA. Wrote the paper: CD MM CVA. Managing and Providing funds for the research: CVA MAB CD MM.

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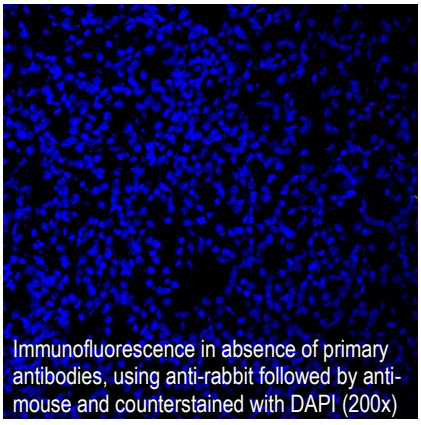
A GRFa2/Prop1/Stem (GPS) Cell Niche in the Pituitary

by Montse Garcia-Lavandeira *et al.*

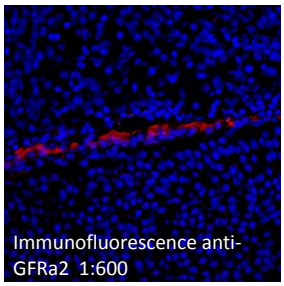
Supplementary Methods

Immunodetection. For immunofluorescence, rat pituitaries were oriented and immersed in an OCT-filled plastic cryomold (Sakura) and frozen inside a glass beaker filled with isopentane previously immersed in liquid N2; frozen cryomolds were maintained at -80ºC until sectioned in 10 microM cryosections. The sections were fixed and processed as described in the main text.

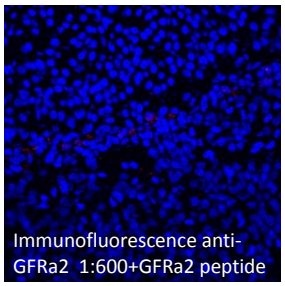
Double immunofluorescences were performed in consecutive days; to prevent secondary antibody backgrounds, the order was dependent on the species of the primary antibody: first day goat, guinea pig or rabbit, second day rabbit or mouse respectively. Negative controls (using PBS instead of primary antibody) were routinely run in parallel (see left picture). Preadsorbed controls, where the antibodies were previously incubated overnight with an excess of the peptide (GFRa2, Abcam), the fusion protein (GST-Prop$_{155-223}$) or the full-length protein (Nanog, Abcam) were also performed; the intensity of the specific signal was practically abolished (see pictures). Nuclei were counterstained with 20 □g/ml DAPI (Sigma). A TCS-SP2-DMRE Confocal Microscope with Ar, He/Ne 543 and He/Ne 633 Lasers (Leica) and LCS software was used to analyze the results.



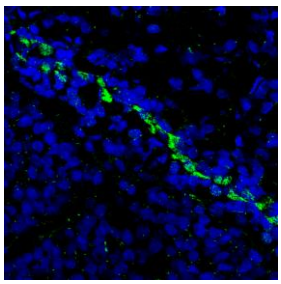
Immunofluorescence in absence of primary antibodies, using anti-rabbit followed by anti-mouse and counterstained with DAPI (200x)

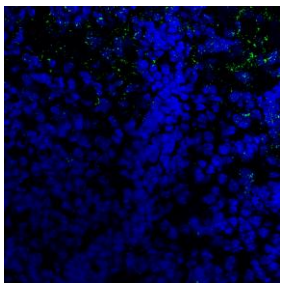


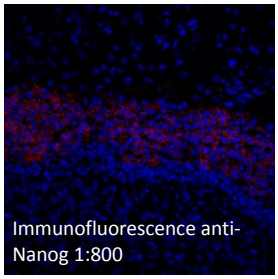
Immunofluorescence anti-GFRa2 1:600

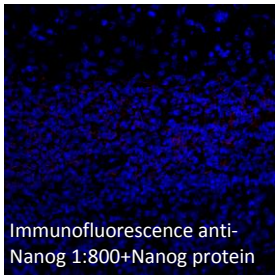


Immunofluorescence anti-GFRa2 1:600+GFRa2 peptide

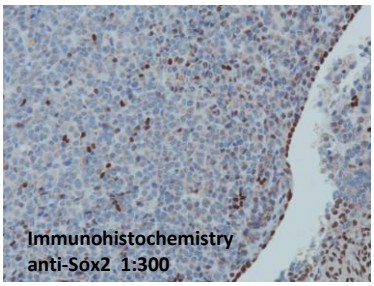


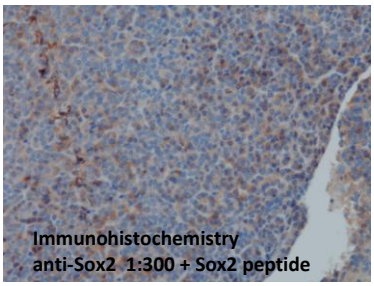






Negative and preadsorption (Sox2 peptide, Chemicon AG980) controls were also used for immunohistochemistry in human and mouse sections.





For a detailed list on antibodies and dilutions see Supplementary Table S1 and S2.

For immunoblotting, tissues o cells were lysed as previously described [4, 5]. Shortly, after adding 1% SDS at 95 ºC during 5 minutes the lysate was diluted 1:5 with 50mM Hepes pH 7.5, 150 mM NaCl, 10% Glycerol, 1% Triton, 5 mM EGTA, 1.5 mM MgCl supplemented with 0.1 M PMSF (Sigma), 5mg/ml Aprotinine (Sigma), 2% Na_3VO_4 (Sigma), 0.1 M Na-pyrophosphate (Sigma), homogenized through a 20 gauge syringe and incubated for 20 minutes on ice. Lysates were spun for 5 minutes at 14000 rpm and supernatants kept at -20 ºC. 75 microg of total proteins were load in 8-15 % SDS-PAGE gels. Incubation with primary antibodies was performed overnight (see Supplementary Table S1 and S2 for dilutions) and alkaline phosphatase-conjugated secondary antibodies and the CSS system (Tropix) were used for immunodetection.

Sorting of GFRa2+ cells. Pituitaries from wild type male mouse were dissected and mechanically minced in PBS-1 (PBS supplemented with 2.7 mg/ml D-glucose, 10 mg/ml BSA, 0.5 mM CaCl2, 1 mM MgCl2). Fragments were further digested with 0.1% Collagenase type IA (Sigma), 0.05% (100 IU/ml) DNAse, 5 mM CaCl2 in PBS for 45 minutes with gentle agitation. After centrifuging, cells were washed for 15 minutes in PBS-2 (PBS supplemented with 2.7 mg/ml D-glucose, 10 mg/ml BSA) with gentle agitation. After this washing, cells were centrifuged and divided in two identical aliquots. The first aliquot was incubated during 45 minutes with 1: 50 anti-GFRa2 in PBS-3 (PBS supplemented with 0.5% FCS and 0.1% NaN3); the second aliquot was maintained in the same volume of PBS-3 with rabbit Ig, without antibody. After the

incubation, and two further washings in PBS-3, both aliquots were incubated during 45 minutes, in the dark and with gentle agitation, with 1:50 chicken anti-rabbit Alexa 488 (Molecular Probes, A21441) in PBS-3. After two last washes in PBS-3, cells were analyzed in a FACScanto (Becton Dickinson).

Isolation and culture of GFRa2+ cells. Freshly isolated cell suspensions were prepared from male rat or mouse pituitaries using magnetic activated cell sorting (MACS; Miltenyi) or a fluorescence-activated cell sorter (FACS; FACSAria, Becton-Dickinson). Murine AP cells were dissected from NP and IL, washed in DMEM (Sigma) and incubated with 0.4% Collagenase type IA (Sigma), 0.05% (100 IU/ml) DNAse I (Sigma) (without/with 0.5% Trypsin, Sigma) in DMEM supplemented with 20% horse serum (HS, Gibco) in the incubator; mechanical dispersion was performed each 5 minutes. After 20 minutes, dispersed cells were washed twice in 20 % HS-DMEM and incubated with rabbit anti-GFRa2 antibody for 30 minutes at room temperature. After this incubation, 1 ml of 0.5% BSA (Sigma), 2mM EDTA in PBS (pH: 7) was added and cells were washed and resuspended in 200 □l of the same buffer plus 50 ml of anti-rabbit Microbeads (Miltenyi) for 15 minutes at 4ºC. After washing, cell-beads suspension was flowed through an MS Column (Miltenyi) attached to the magnetic separator (MiniMACS), with two 500 □l washings (GFRa2- cells). GFRa2+ cells were eluted in 1 ml of media and cultured as follows (all the experiments with spheroids shown were performed with rat cells, although many have been reproduced in mouse cells, data not shown):

a) **<u>As spheroids:</u>** GFRa2+ cells were eluted in 1 ml of **SpherM:** 1x N2 (Invitrogen), 1x B27 (Invitrogen), 100 IU/ml penicillin, 100 □g/ml streptomycin, and 2.5 □g/ml amphotericin-B in 1:1 DMEM/F12 (vol/vol) medium. 2500 cells were cultured in a p35 dish. After 5-7 days spheroids were either video-recorded or fixed for further immunofluorescence or induced to differentiate. For the BrdU-uptake experiments, 10 microM BrdU (Sigma) was added from the beginning, but a toxic effect was seen with longer treatments than 5 days; to evaluate the % of cell division in spheroids of different days BrdU was added for the last 12 hours of incubation before fixation. To study clonality, e.g. that the spheroids originated from a single cell, cells were also seeded in lower dilutions 20-18 cells/ml in 24-well dishes, and further diluted 1:2 in the following columns up to 0,5 cells/ml. After five days all the spheroids were photographed (Supplementary Figure 3A).

To induce differentiation, every individual spheroid was carefully pipetted under the microscope at day 5, isolated and placed in Collagen type IV or poly-L-lysine treated Cultureslides (BD) in 10 % FCS-SpherM. The following day, the medium was replaced during 15 days by one of the following differentiation media (**DifM**):

-DifM1: 1:1 SSM+ conditioned medium from the rat-pituitary cell line GH4C1 (vol/vol) supplemented with antibiotics. The **SSM** or Semisynthetic medium has been previously described by our group for primary pituitary culture [4-6] [6,5:3,5 DMEM/Ham's F12 medium (vol/vol) supplemented with (Sigma): (per liter) BSA (2 g), HEPES (2.38 g), hydrocortisone (143 □g), T3 (0.4 □g), Transferrin (10 mg), Glucagon (10 ng), Epidermal growth factor (0.1 □g), and Fibroblast growth factor (0.2 □g)]. The **conditioned medium from the GH4C1** was obtained by culturing the cell-line in DMEM+10%FCS; after 6 days without change, the medium was collected, filtered and frozen in aliquots.

-DifM 2: 1: 1 DMEM/Ham's F12 (1:1), 1x N2 + conditioned medium from the rat-pituitary cell line GH4C1 (vol/vol) supplemented with antibiotics.

-DifM 3: 1x N2, 1% FBS in SSM supplemented with antibiotics.

-DifM 4: 1x B27, 0,5 ng/ml FGF, 10-9 M GHRH, 10-9 M Ghrelin, 10-9M Somatostatin, 10-9M Hydrocortisone, 5 microg/ml Transferrin, 10 microg/ml Insulin in 1:1 DMEM/Ham's F12 (vol/vol) supplemented with antibiotics.

-DifM5: 1:1 SSM+ conditioned medium from the rat-pituitary cell line GH3 (vol/vol) supplemented with antibiotics. The **conditioned medium from the GH3** was obtained by culturing the cell-line in DMEM+2,5%FCS+15% Horse Serum; after 6 days without change, the medium was collected, filtered and frozen in aliquots.

b) **<u>As attached cell culture:</u>** GFRa2+ were eluted in 1 ml of **MEFM** and seeded in gelatin-coated wells [**MEFM: 1:1 StemM + conditioned medium from MEFs (vol/vol). StemM** (Millipore): 1% non-essential aminoacids, 2mM Glutamine, 0,1mM b-mercaptoethanol, 20% serum-KO replacement, 80% KO-DMEM supplemented with antibiotics. **Conditioned medium from MEF:** mouse embryonic fibroblasts were passaged in DMEM containing 20% FBS and treated with mytomicin. To obtain Conditioned medium, they were incubated in StemM (1% non-essential aminoacids, 2mM Glutamine, 0,1 mM b-mercaptoethanol 20%

serum-KO replacement, 80% KO-DMEM supplemented with antibiotics); after 6 days without change, the medium was collected, filtered and frozen in aliquots.

The cells were passed every 25 days but we observed that many cells differentiated on the dish in these conditions.

c) **<u>As floating spheres:</u>** When cells were cultured in MEFM plus 1000 U/ml Esgro (Chemicon) (**MEFM-E**) they did not attach to the coated dish nor differentiate; they grew as groups of cells floating in the medium. They grew slowly but steady and were passed every 25 days, by centrifugation, pipet-dispersion and dilution 1:3.

d) **<u>As embryonic-like colonies:</u>** MEF fibroblasts were treated with mitomycin as described and frozen in aliquots. When required, an aliquot was thawed and fibroblast seeded as a monolayer. The next day, GFRa2+ purified cells were eluted in **StemM** and overlaid on top of the MEF monolayer. Two-week later colonies were formed. Attached or floating Colonies were routinely passed every 25 days with the help of Accutase (Chemicon).

Immunofluorescence of spheroids was performed pipetting them on top of 8 □m inserts (Millipore) and fixing them with 70 % Ethanol at room temperature during 30 minutes, plus 4M HCL during 20 minutes (BrdU labeling) or with -20 ºC Methanol for 5 minutes for the other antibodies before proceeding as above. Differentiated cells were fixed in Methanol (hormones) or in 4 % paraformaldehyde for 20 minutes (hormones, Tubulin-beta-III, NF). (See below for antibodies and dilutions).

Confocal quantitative telomere FISH (Telomapping). For telomapping, paraffin-embedded tissue sections were hybridized with a PNA-tel Cy3-labelled probe and telomere length was determined as described [7]. Slides were deparaffinized in three xylene washes (3 minutes each), then treated for 3 minutes with a 100, 95 and 70% ethanol series, followed by telomere Q-FISH protocol performed as described [8]. DAPI, Cy3 signals were acquired simultaneously into separate channels using a confocal ultraspectral microscope (Leica TCS-SP2-A-OBS-UV) using a PL APO 20x/0.70 PH2 as lens with Leica LCS software and maximum projections from image stacks (10 sections at steps 1.0 microM) were generated for image quantification. The DPSS-561 laser (Cy3 laser) was hold at a constant intensity to capture all the mouse tissues images. Highthoughput quantitative image analysis was performed on confocal images using the Metamorph platform (version 6.3r6; Molecular Devices, Union City, CA).

The DAPI image was used to define the nuclear area and the Cy3 image to quantify of telomere fluorescence. In all cases, background noise was subtracted from each image prior to making qualitative measurements. The DAPI images were signal-intensity thresholded, segmented and converted to 1-bit binary image. The binary DAPI mask was applied to the matching Cy3 to obtain a combined image with telomere fluorescence information for each nucleus. Cy3 fluorescence intensity (telomere fluorescence) was measured as "average gray values" (total gray value/nuclei area) units (arbitrary units of fluorescence). These "average telomere fluorescence" values always represent the average Cy3 pixel intensity for the total nuclear area, and not the average value of individual telomere spot intensities, therefore ruling out that differences in nuclear size may influence telomere length measurements. A code of four colors was used to classify the nuclei according to their average telomere fluorescence. Finally, telomere fluorescence values for each histological region were exported to Excel and the frequency histograms were generated.

Immunolabeled cell counting on paraffin sections

Mouse pituitaries were embedded in paraffin in either sagittal or coronal orientation. A Hematoxylin staining was performed in sections every 50 microM to detect the biggest section per pituitary. Immunohistochemistry was performed in three sections around the middle separated by at least 20 microM. A minimum of 3 pituitaries per orientation and 250 cells per mice were scored (at least two males and two females). The number of cells per area was counted in sections. On the other hand, the volume of the pituitary was calculated using the following equation (volume of an ellipsoid):

$$\text{Pituitary volume} = (\text{Major axis} \times \text{Minor axis}^2)/2$$

(as mean among four mice per group).

qRT-PCR primers and conditions. Total RNA from male rat pituitaries was extracted with RNeasy Mini Kit (Quiagen). 1 □g RNA was reverse-transcribed using the standard protocol for MMLV (Invitrogen) in 20 □l reaction. 3 □l of the reaction were amplified with Cybergreen PCR mix (Roche) in a 7300 TaqMan (Applied), using the following conditions: 95ºC 2", 60ºC 15", 72ºC 15". A list of the Oligonucleotides used is shown in Supplementary table S3.

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Supplementary Table S1. List of Antibodies and dilutions. Primary Antibodies

Protein	Species	Catalog Nº	Company	Dilution			
				Tissue	Spheroid	Differentiation	WB
GFRa2	Rabbit	AB5141	Chemicon	1:500	1:50	1:50	1:600
GH	Rabbit	AFP-C11981A	NIDDK	1:800	1:300	1:300	1:1000
GH	Guinea pig	AFP 222 387 790	NIDDK	1:800			
ACTH	Mouse	M3501	Dako	1:100		1:50	
PRL	Mouse	E30610M	Biodesign	1:200	1:100	1:100	1:1000
beta-TSH	Goat	sc-7813	Santa Cruz B	1:200			
beta-TSH	Rabbit	AFP-1274789	NIDDK			1:50	
beta-FSH	Mouse	18-0020	Zymed Laboratories	1: 75		1:50	
beta-LH	Mouse	MS-9078-P	Neo Markers	1:250			
Cytokeratins	Mouse	NCL-C11	NovoCastra	1:45			
E-Cadherin	Mouse	C20820-050	BD biosciences	1:75	1:50		
b-Catenin(IF)	Mouse	05-665	Upstate	1:300	1:50		
b-Catenin(IHQ)	Rabbit	sc-1496	Santa Cruz B	1:200			
Oct4	Mouse	MAB4305	Chemicon	1:100	1:50		
SSEA-4	Mouse	MC-813-70	Hybridoma bank	1:50			
Nanog	Rabbit	Ab5731	Chemicon	1:800			
Prop1	Guinea pig	Against GST-Prop$_{155-230}$ and His-Prop$_{155-230}$	in house A.K. Ryan	1:250	1:50	1:50	1:500
S100 (rat)	Rabbit	RTU-RTUS100p	Novocastra	Commercially pre-diluted			
S100 (mouse, human)	Rabbit	Z0311	Dako	1:100 (mouse) 1:2000 (human)			
Vimentin (rat)	Rabbit	(H-84): sc-5565	Santa Cruz B	1:200			
Vimentin (mouse)	Guinea Pig	RDI-PROGP53	Fitzgerald	1:25 (Prot K)			
Vimentin (human)	Mouse	clone V9 M0725	Dako	1:500 (Citrate)			

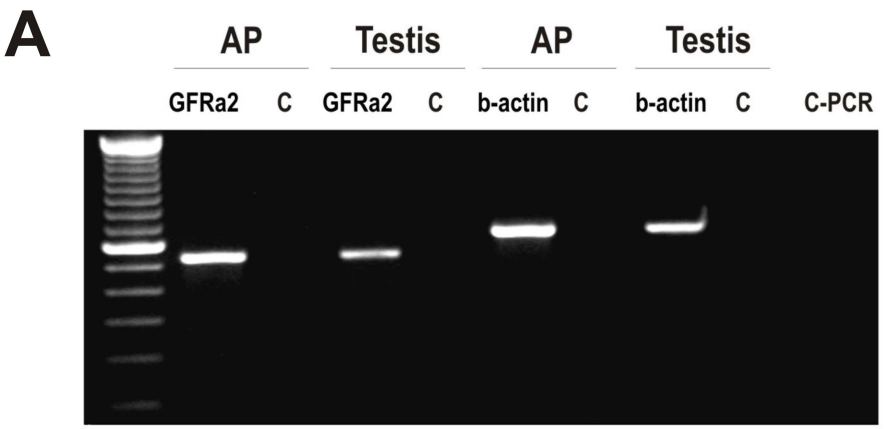
Nestin	Mouse	Rat-401	Hybridoma bank	1:100			
Ki67(rat)	Mouse	M7248	Dako	1:250			
Ki67(mouse)	Mouse	000310Q10(SP6)	Master D	1:200			
Sox2	Mouse	MAB4343	Chemicon	1:100			
Sox2	Rabbit	AB5603	Chemicon	1:300 (IHQ) 1:500 (IF)			
Sox9	Rabbit	AB5535	Chemicon	1:200 (IHQ) 1:500 (IF)			
Sox4	Rabbit	S7318	Sigma	1:50			
NTN	Goat	AF477	R&D systems	1:100			1:500
Ret	Goat	sc-1290	Santa Cruz B	1:100			
Pit1	Rabbit	sc-442	Santa Cruz	1:300			
beta-Tubulin isotype III	Mouse	T8660	Sigma			1:100	
Neurofilament (PAN)	Mouse	FNP7,DA2,RmdO2011	Zymed			Commercially pre-diluted	

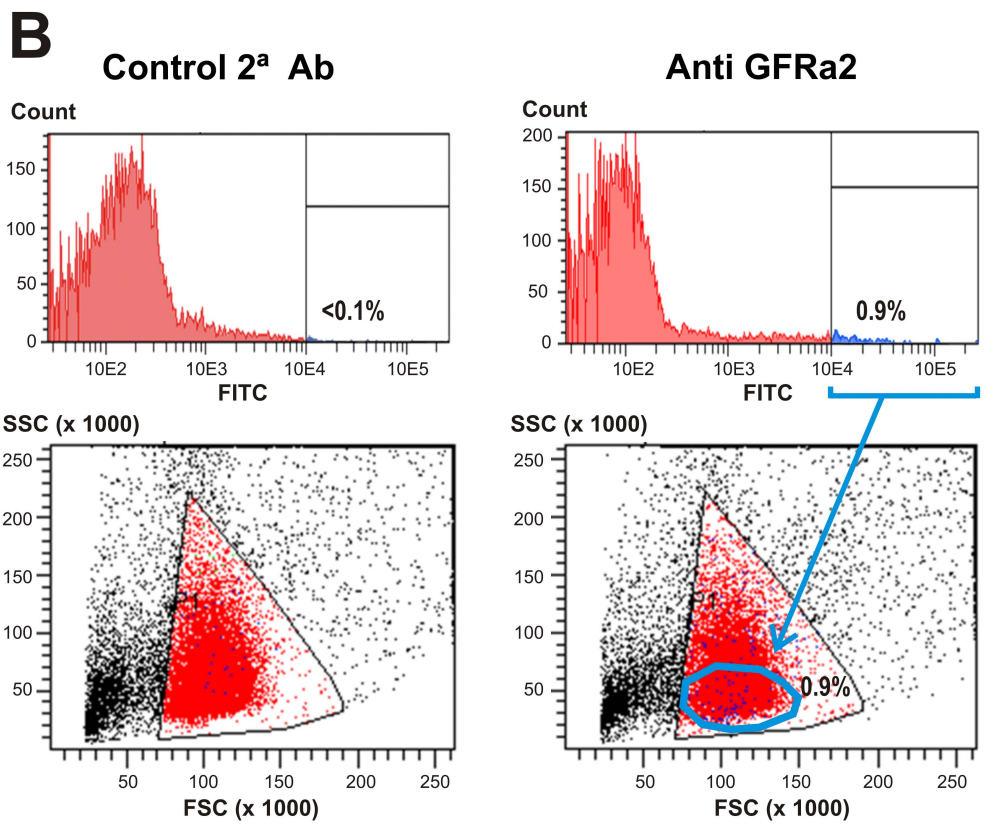
Supplementary Table S2. List of secondary antibodies and related reagents.

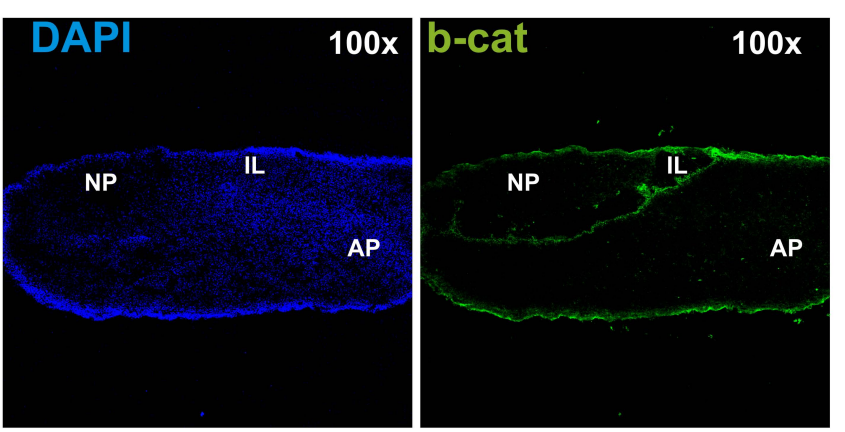
Secondary antibody	Labeling	Catalog number	Company	Dilution			
				Tissue	Spheroid	Differentiation	WB
Rabbit anti-Mouse	Cy™2	315-226-047	Jackson InmunoResearch	1:1000	1:400	1:400	
Goat anti-Rabbit	Cy™3	111-166-047	Jackson InmunoResearch	1:1000	1:600	1:600	
Donkey anti-Goat	Alexa Fluor 488	A-11055	Molecular Probes	1:250			
Goat anti-Guinea Pig	Alexa Fluor 488	A-11073	Molecular Probes	1:1500	1:600		
Goat anti-rabbit	Botinylated	BA-100	Vector Labs	1:200			
Goat anti-mouse	Biotinilated	BA-200	Vector Labs	1:200			
Goat anti-Rabbit	Alcaline phosphatase phosphatase	AC31RL	Tropix				1:5000
Goat anti-Mouse	Alcaline phosphatase phosphatase	AC32ML	Tropix				1:5000
Protein A	HRP	NA9120V	Amersham				1:5000

Supplementary Table S3. Oligonucleotides used to analyze gene expression by RT-PCR.

Genes	Forward (F) and Reverse (R) Primers.	Fragment (bp)
GRFa2	F-5´TTCAGGCTCGCTTCAATCTT 3´ R-5´AACTGCAAGAAGCTTCGCTC 3´	122
Oct4	F-5´CAAGTTGGCGTGGAGACTCTGC 3´ R-5´AGACAACCATCTGCCGCTTCG 3´	299
Prop1	F-5´GACAGCTGGAGTCAGCCTTT 3´ R-5´GCAAGAGCGGTCACTACTCC 3´	153
GH	F-5´GCAGAGAACTGACATGGAATTG 3´ R-5´ GATGACGCTCTGCTCAAAA 3´	269
Ret	F-5´ CATCAAGTTGTACGGGGCTT 3´ R-5´ TGCCTCCACTGCTCACATAG 3´	134
Pit1	F-5´TTCCAGACCACACCCTGAGT 3´ R-5´ACTTTTCCGCCTGAGTTCCT 3´	190
Hprt	F-5´CAGTCCCAGCGTCGTATT 3´ R-5´AGCAAGTCTTTCAGTCCTGTC 3´	139

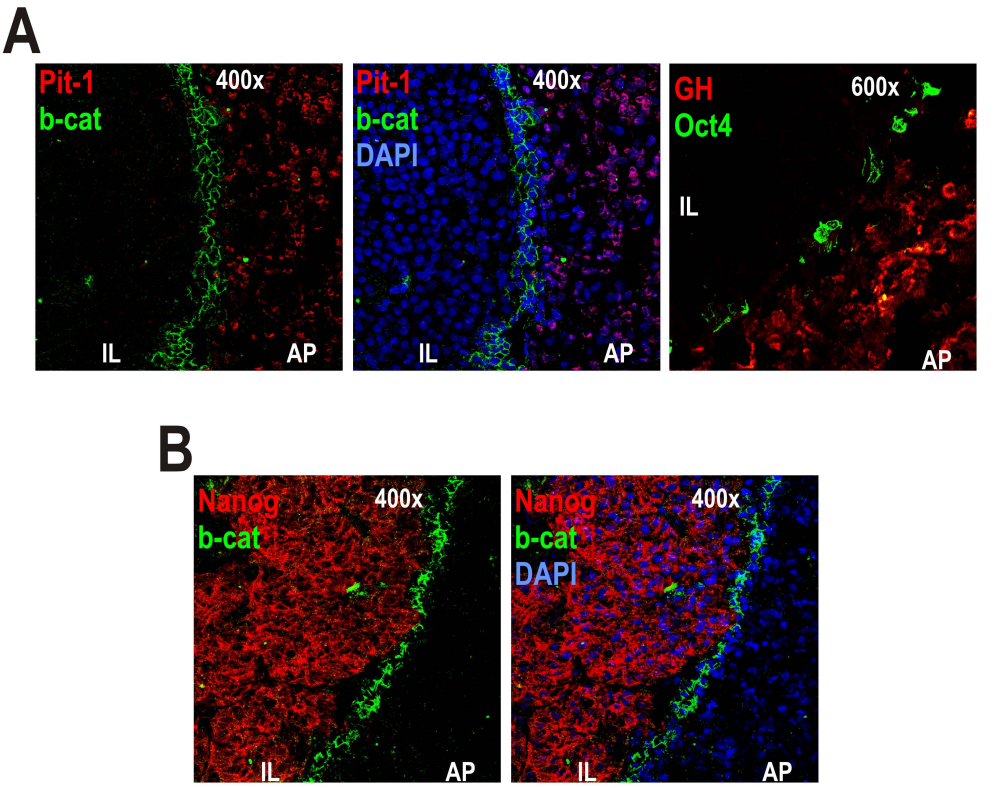




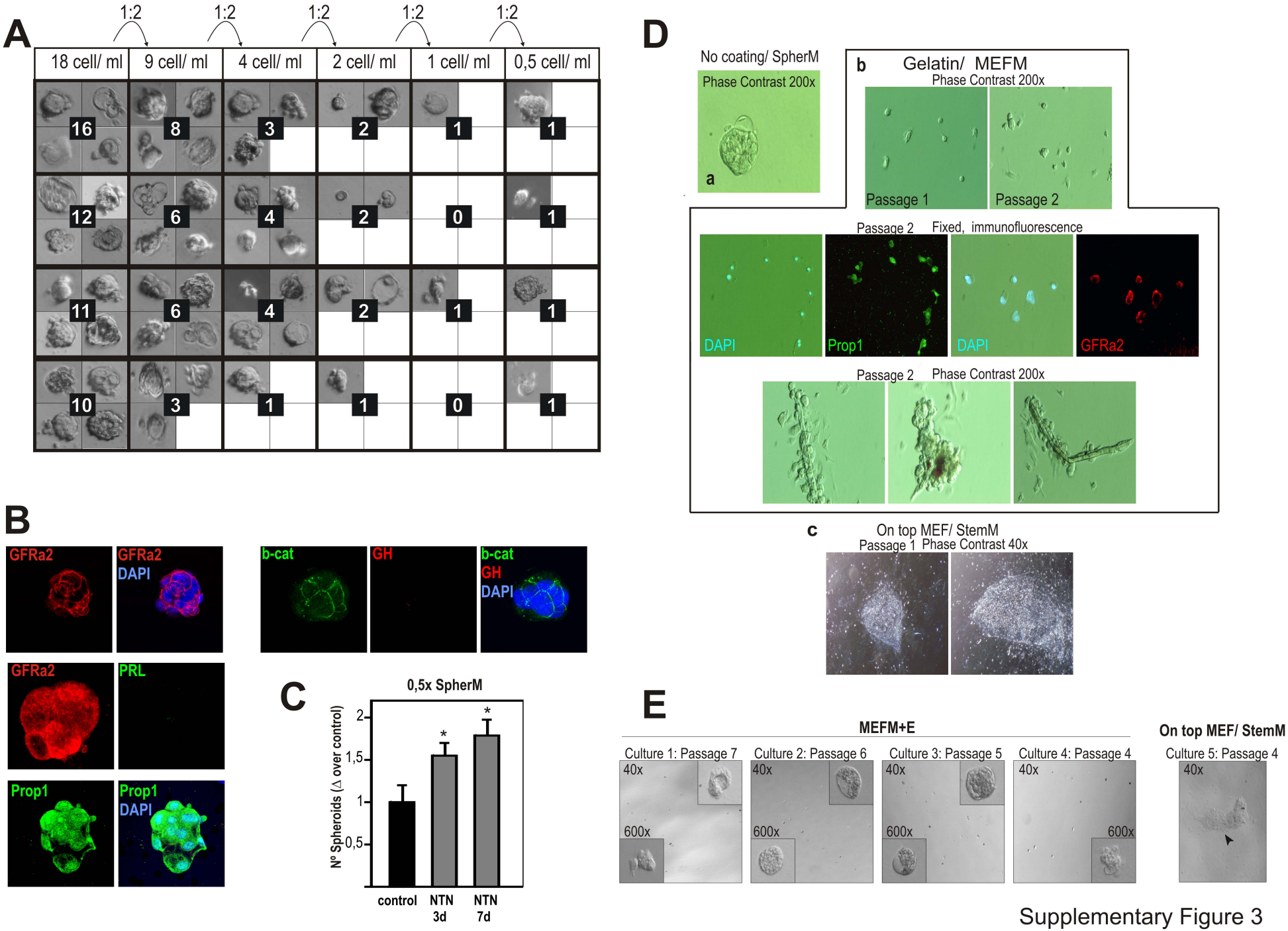


Pituitary 10 days

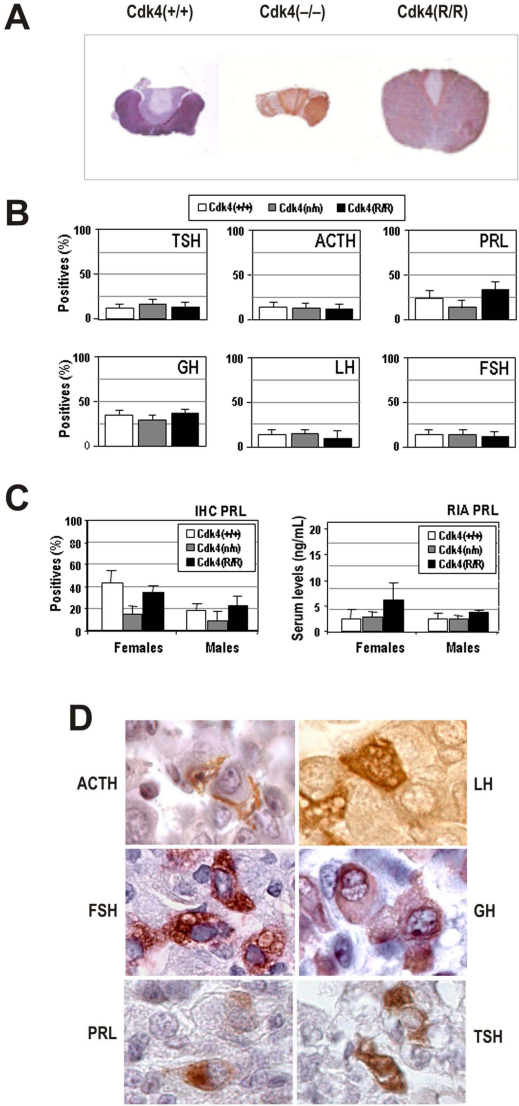
Supplementary Fig 1



Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4

3.4.- Las familias de inhibidores INK4 y Cip/Kip previenen el estrés replicativo durante el desarrollo

Las familias de inhibidores de ciclo celular Cip/Kip e INK4 están involucradas en senescencia celular y supresión tumoral, aunque recientemente un posible papel oncogénico ha sido descrito para los inhibidores de la familia Cip/Kip, $p21^{Cip1}$ y $p27^{Kip1}$. Para comprender el papel de estos inhibidores y un posible efecto sinérgico, hemos caracterizado animales carentes de $p21^{Cip1}$ y p27Kip1 que expresan de manera endógena el mutante Cdk4 R24C [alelo Cdk4 (R)], que hace a esta proteína insensible a la inhibición por proteínas de la familia INK4. Hemos comprobado que combinaciones intermedias de los alelos Cdk4(R); p21(–);p27(–) mueren debido a la aparición de tumores principalmente de carácter endocrino, aunque también en algunos genotipos de angiosarcomas. Conforme vamos introduciendo alelos de p27(–) las latencias se vuelven más cortas, mientras que la introducción de alelos p21(–) sólo tiene un efecto moderado en la vida media de los animales. A medida que va disminuyendo la latencia, la variedad tumoral se va restringiendo llegando a hacerse exclusiva de tumores de hipófisis. Estos tumores presentan variaciones en su origen conforme se incrementa la agresividad del genotipo, llegando a resultar imposible su clasificación histopatológica. Animales homocigotos para las tres mutaciones mueren, sin embargo, de manera perinatal presentando una hipoplasia generalizada. Esta hipoplasia se caracteriza por un incremento en el número de células ciclando y un aumento en la presencia de marcadores de daño al DNA (gammaH2AX, p53) que resulta en un incremento en la apoptosis.

Quereda V, Dubus P, Cañamero M, Temple S and Malumbres M. 2010. Ink4 and Cip/Kip family inhibitors prevent replicative stress during development. (Manuscript in preparation)

Contribución del doctorando al trabajo

Victor Quereda realizó los todos los experimentos que se presentan en el artículo. Se encargó del mantenimiento de la colonia de animales, organizando los cruces y realizando

las necropsias a los animales que aparecen en las diferentes gráficas. Colaboró en el diseño de los experimentos y en la escritura del artículo.

Ink4 and Cip/Kip Cell Cycle Inhibitors Cooperate in Preventing Replicative Stress during Development

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Abstract

Cell-cycle inhibitors of the INK4 and Cip/Kip families are involved in cellular senescence and tumor suppression. The inactivation of individual members of these families frequently results in tumor development. To understand the relevance of the combined activity of INK4 and Cip/Kip inhibitors, we have characterized a mouse model which combines the absence of $p21^{Cip1}$ and $p27^{Kip1}$ proteins with the endogenous expression of Cdk4 R24C mutant that is insensitive to INK4 inhibitors. The accumulation of Cdk4 R24C, p21-null and p27-null alleles results in frequent hyperplasias and tumor development, mainly in cells of endocrine origin such as pituitary cells. The inactivation of $p27^{Kip1}$ strongly cooperates with Cdk4 R24C whereas the presence of $p21^{Cip1}$ mutant alleles only affects modestly tumor latency in the Cdk4 R24C background. Complete abrogation of $p21^{Cip1}$ and $p27^{Kip1}$ in Cdk4 R24C mutant mice results in perinatal death. Interestingly, these triple mutant mice display reduced size and massive hypoplasia. This phenotype correlates with increased DNA damage in multiple tissues and the subsequent apoptotic cell death. These data suggest that cell cycle inhibitors cooperate to prevent replicative stress in primary cells and during development.

Introduction

The involvement of cell cycle regulators in human cancer has been extensively established in the last years (Malumbres & Barbacid, 2001; Sherr, 2000). A vast number of these alterations have been reported in the cell-cycle regulators that control the entry into the cell cycle from quiesce and the G1/S transition. The retinoblastoma protein (pRb) pathway seems to play a key role in the regulation of these cellular processes since pRb and their regulators —cyclins, cyclin-dependent kinases (Cdks) and Cdk inhibitors— are frequently mutated in human cancer (Malumbres & Barbacid, 2001).

In normal cells, pRb proteins repress the transcription of genes required for DNA replication or mitosis and maintain cells in a quiescent state. This function is achieved through the sequestering of inactive E2F transcription factors and through the binding to histone deacetylases and chromatin remodeling complexes. Upon mitogenic stimuli, D-type cyclins are induced and activate the cell cycle kinases Cdk4 and Cdk6. Cyclin D-Cdk4/6 complexes phosphorylate and partially inactivate pRb, allowing the expression of some E2F-target genes such as cyclin E. Induction of

Table 1. Tumor susceptibility in Cdk4 R24C; p21KO; p27KO mutant mice

	(+/+)(+/+)(+/+)	(R/R)(+/+)(+/+)	(R/R)(-/-)(+/+)	(+/R)(+/-)(+/-)	(+/R)(-/-)(+/-)	(R/R)(+/-)(+/-)	(R/R)(-/-)(+/-)	(+/R)(+/-)(-/-)	(+/R)(-/-)(-/-)	(R/R)(+/+)(-/-)	(R/R)(+/-)(-/-)
N	23	17	19	6	17	25	20	6	10	7	10
Angiosarcoma	0,0%	56,0%	47,0%	50,0%	35,3%	20,0%	25,0%	0,0%	0,0%	0,0%	0,0%
Osteosarcoma	0,0%	1,0%	11,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
Squamous cell	0,0%	0,0%	5,0%	0,0%	0,0%	0,0%	5,0%	0,0%	0,0%	0,0%	0,0%
Pancreatic endocrine	0,0%	31,0%	21,0%	0,0%	0,0%	20,0%	0,0%	0,0%	0,0%	0,0%	0,0%
Pituitary	0,0%	22,0%	21,0%	83,3%	53,3%	96,0%	60,0%	83,3%	50,0%	100,0%	90,0%
Lymphoid	5,0%	2,0%	7,0%	0,0%	0,0%	4,0%	0,0%	0,0%	0,0%	0,0%	0,0%
Phechronocitoma	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
Lung	0,0%	18,0%	11,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
Prostatic neoplasia	0,0%	0,0%	0,0%	0,0%	0,0%	4,0%	0,0%	0,0%	0,0%	0,0%	0,0%
Leydig cell tumour	0,0%	0,0%	0,0%	0,0%	17,6%	24,0%	10,0%	0,0%	0,0%	0,0%	0,0%
Animals without tumour	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	20,0%	16,7%	50,0%	0,0%	10,0%
Total Incidence	4,00%	94,00%	100,00%	100,0%	100,0%	100,0%	100,0%	100,0%	100,0%	100,0%	100,0%
Average Latency	>100	69	53,7	59,3	55,2	37,7	33,1	19,3	17,2	7	6,8

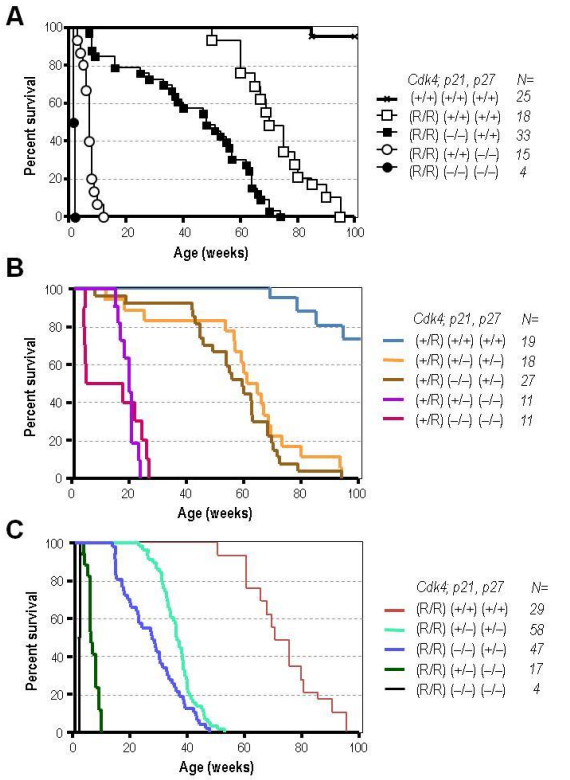

FIG. 1. Survival in *Cdk4 R24C; p21KO ;p27KO* mice colony. Genotypes as indicated (A) Survival of homozygous mice for the different alleles in our colony of Cdk4; $p21^{Cip1}$ and $p27^{Kip1}$ mice. (B) Comparative representation of $p21^{Cip1}$ vs. $p27^{Kip1}$ survival curve in a *Cdk4(+/R)* background. (C) Survival of $p21^{Cip1}$ vs. $p27^{Kip1}$ in *Cdk4(R/R)* background

Cyclin E may allow the activation of Cdk2 which is also able to further phosphorylate pRb. Cdk2 is also able to bind A-type cyclins during S phase, whereas the control of G2 and M phases mainly depends on cyclin A- and cyclin B-Cdk1 complexes (Malumbres & Barbacid, 2005).

Whereas mitogenic stimuli induce cyclins and therefore activate Cdks, antimitogenic signals arrest this process by inducing members of the two families of Cdk inhibitors (CKIs), the INK4 and Cip/Kip families (Sherr & Roberts, 1999). The inhibitors of the INK4 family, formed of $p16^{Ink4a}$, $p15^{Ink4b}$, $p18^{Ink4c}$ and $p19^{Ink4d}$, specifically bind *Cdk4* and *Cdk6* in order to inhibit its catalytic activity. On the other hand, Cip/Kip family members, $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$, are able to bind and inhibit several Cdk-Cyclin complexes.

Resistance to Ink4 inhibitiors using the Cdk4 R24C mutation in a knockin model has been shown to have tumorigenic activity confirming the tumor suppressor role of these inhibitors (Sotillo et al, 2001a; Sotillo et al, 2001b). On the other hand, individual ablation of either $p21^{Cip1}$ or $p27^{Kip1}$ result in increased susceptibility to tumor development (Fero et al, 1996; Kiyokawa et al, 1996; Martin-Caballero et al, 2001; Nakayama et al, 1996). The Cdk4 R24C mutation cooperates with lack of either $p27^{Kip1}$ or $p21^{Cip1}$ in the development of pituitary tumors or sarcomas, respectively (Quereda et al, 2007; Sotillo et al, 2005). To investigate the relative roles of INK4 and Cip1/Kip inhibitors in tumor appearance and brain development, we have now analyzed the synergistic effect of combining the insensitivity to INK4 proteins (conferred by the Cdk4 R24C mutation) and the $p21^{Cip1}$ and $p27^{Kip1}$ deficiency. Mice of intermediate genotypes display high tumor susceptibility which increase in aggressiveness and decrease in latency together with the depletion of the different alleles. Interestingly, genetic combination of these three alterations of Cdk4 R24C *knock in*, $p21^{Cip1}$ and $p27^{Kip1}$-*null* [*Cdk4*(R/R); *p21*(–/–); *p27*(–/–)] results in perinatal lethality due to a severe proliferative defects in the brain and in the pituitary gland.

Results

Genetic cooperation between $Cdk4^{R24C}$ and lack of Cip/Kip inhibitors

We have analyzed the effect of the combined ablation of $p21^{Cip1}$ and $p27^{Kip1}$ in a Cdk4 R24C background using triple mutant mice. The combination of mutant alleles displayed diverse effects in survival and tumor development in these mouse models. As reported previously, lack of $p27^{Kip1}$ strongly cooperated with *Cdk4* R24C whereas the absence of $p21^{Cip1}$ resulted in a moderate but significant decrease in survival in the *Cdk4 R24C* background [Fig. 1A and (Quereda et al, 2007; Sotillo et al, 2001a; Sotillo et al, 2005)]. The combined ablation of $p21^{Cip1}$ and $p27^{Kip1}$ led to perinatal lethality in *Cdk4*(R/R) mice (average lifespan = 2 weeks; Fig. 1A) whereas $p21^{Cip1}$;$p27^{Kip1}$ double *knock-out* mice are viable and develop similar pathologies to single mutants in a *Cdk4*-wt background (data not shown). An overview to the survival of these mutant mice indicates that loss of 1 or 2 alleles of $p27^{Kip1}$ result in a dramatic reduction in the lifespan of *Cdk4*(+/R) or *Cdk4*(R/R) mice, whereas loss of $p21^{Cip1}$ alleles has a moderate effect (Fig. 1B,C). For instance, whereas the presence of a single *p27*(+) allele [in a *Cdk4*(R/R); *p21*(–/–) background] results in an average lifespan of 30 weeks, the presence of a single *p21*(+) allele only extents the survival of *Cdk4*(R/R); *p27*(–/–) mice a few weeks (lifespan = 6 weeks in *Cdk4*(R/R); *p21*(+/–); *p27*(–/–) versus 2 weeks in *Cdk4*(R/R); *p21*(–/–); *p27*(–/–) mice].

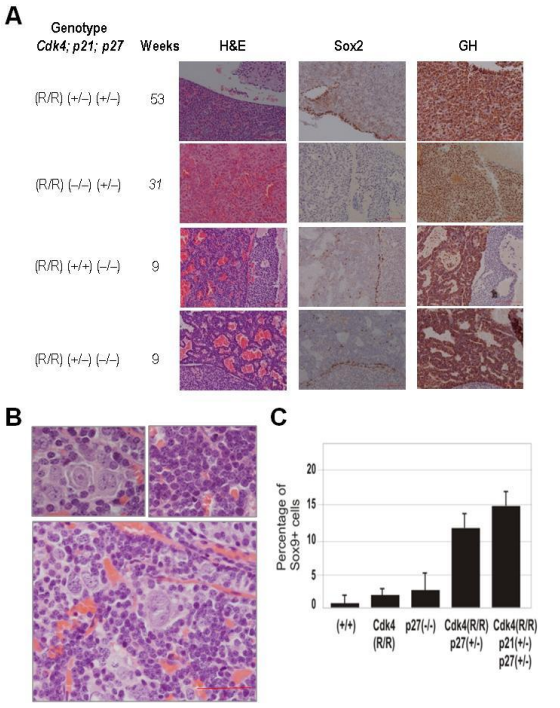

FIG 2. Summary of defects observed in tumoral pituitary glands. (**A**) Representative pictures of H&E, Sox2 and GH from different pathologies found in mutant animals. Genotype and age of dead is indicated. Scale bar represents 500 μm. (**B**) Lower panel represent typical cells found in more undifferentiated pituitary tumors with big neuron-like cells and small cells non-endocrine ones, shown in higher magnification in upper panels. Scale bar represents 100 μm, magnifications are 2.5 X. (**C**) Quantification of Sox9-positive in cells in the pars distalis of the indicated genotypes

Histopathological analysis of these compound mice revealed diverse pathologies whose incidence and latency depends on the genotype (Table 1). Angiosarcomas and pituitary tumors are the most prevalent pathologies in these animals although single or double mutant also develop other epithelial or mesenchymal tumors. As lifespan of these models decrease, pituitary tumors stand as the most prevalent pathology and it is the only tumor observed in those genotypes whose lifespan is lower than 20 weeks of life [*Cdk4*(+/R); *p21*(–/–); *p27*(+/–); *Cdk4*(+/R); *p21*(–/–); *p27*(–/–); *Cdk4*(R/R); *p21*(+/+); *p27*(–/–) and *Cdk4*(R/R); *p21*(+/–); *p27*(–/–)]. Although the development of pituitary tumors is led by the *Cdk4*(R) and *p27*(–) alleles, lack of $p21^{Cip1}$ also cooperates in the development of this pathology in the presence of wild-type alleles of either *Cdk4* or $p27^{Kip1}$. These pituitary tumors are usually accompanied with hyperplasia of other endocrine tissues, such as the endocrine pancreas or adrenal gland, as well as hyperplasia of Leydig cells in the testis (Table S1 and Figure S1).

Interestingly, loss of both $p21^{Cip1}$ alleles prevents pituitary tumors in *Cdk4*(R/R); *p27*(–/–) mice as triple mutant mice die in 2 weeks without tumoral or even hyperplastic pituitaries (see below).

Loss of cell cycle inhibitors results in undifferentiated pituitary neoplasias

The pituitary gland is frequently altered in multiple mouse models of cell cycle deregulation (Quereda & Malumbres, 2009). The pituitary abnormalities in Cdk4 R24C; $p21^{Cip1}$; $p27^{Kip1}$ mutant mice are summarized in Tables 1 and S2. Lack of $p27^{Kip1}$ results in pars intermedia tumors whereas the activating R24C mutation in Cdk4 results in tumors in the pars distalis (Sotillo et al, 2001a). Combination of the $Cdk4^{R24C}$; *p21*(–) and *p27*(–) alleles results in a variety of pituitary pathologies affecting both localizations. In fact, the more aggressive combination of mutant alleles [*Cdk4*(R/R); *p21*(+/+); *p27*(–/–) and *Cdk4*(R/R); *p21*(+/–); *p27*(–/–)] results in about 70% of undifferentiated pituitary neoplasias whose cell-of-origin is difficult to assess. In some cases, these tumors also presented regions that were positive for hormone markers such as Growth hormone (GH), FSH or prolactin (Fig. 2A and Table S2).

Undifferentiated pituitary tumours are frequently heterogeneous and hemorrhagic with regions of small sized cells and regions of big, neuron-like cells (Fig. 2B). The small sized cells regions are usually positive for the progenitor markers Sox2 or Sox9. These markers have been recently proposed to be expressed in pituitary progenitor cells that are frequently lined into a single raw of cells in the PD/PI border (Fauquier et al, 2008; Garcia-Lavandeira et al, 2009). The presence of undifferentiated and Sox-positive cells progressively increases as more mutant alleles are present in these animals (Fig. 2C). The presence of differentiated hormone producing cells in the pituitary tumors, the shorter latencies of most of these tumours and the impossibility to localize the origin of the tumor due to its size and localization in the pituitary made us thought on a stem cell origin of the tumor. To address this possibility, fifty thousand (data not sound) or five hundred thousand cells GFRa2-positive and negative were isolated from tumors using MACS technology and reimplanted in the cranial cavity of SCID mice (fig 2S). GFRa2 is a surface marker described to be present only in the putative stem cells of the pituitary gland (Garcia-Lavandeira et al, 2009). When this protocol was performed, pituitary-like tumors appeared in both animals injected with GFRA2 negative and positive cells with the same latencies (fig 2S D) and the pathological characteristics of the tumors was coincident among them and to the original tumors with presence of Sox9 positive cells and an

undifferentiated pattern of the tumor (fig 2S B, C). Serial transplation was performed from pituitary-like tumors arisen from GFRa2 positive injected animals. Coincidently with the first injection experiment, no differences were observed in the apparition or agressivity of the secondary or in the terciary tumours. The impossibility to determine these GFRA2-postive cells as the tumor stem cell population may be due to either they weren't these tumor stem cells or to the fact that the negative population was so aggressive that they don't need big amounts of cells to reproduce the tumor.

Inactivation of Ink4, $p21^{Cip1}$ and $p27^{Kip1}$ inhibitors results in perinatal lethality in the absence of tumors

Despite the frequency of tumors in most compound genotypes, the complete absence of $p21^{Cip1}$ and $p27^{Kip1}$ inhibitors in a $Cdk4^{R24C}$ background results in early lethality in the absence of tumors or obvious hyperplasia. *Cdk4*(R/R); *p21*(–/–); *p27*(–/–) mice were born at the expected mendelian ratio and they had normal appearance as compared with their control littermates at the moment of the birth. However, by 2 weeks of age, they presented a significantly reduced weight compared with their littermates (4 g vs. 8.5 g Fig. 3A) and presented reduced mobility. These phenotypes became more evident in the following few days and *Cdk4*(R/R); *p21*(–/–); *p27*(–/–) mice died before the third week of age. Histopathological analysis of these animals revealed several developmental defects basically represented by massive hypoplasia as exemplified in bone marrow with very low percentage of lymphoid and megakaryocytic cells, or in testis, with a significant decrease in the number of Leydig cells. The mice also presented very congestive arteries (Fig. 3B).

Interestingly, no pituitary hyperplasia was observed as it could be expected from their related genotypes. Instead, the pituitary of *Cdk4*(R/R); *p21*(–/–); *p27*(–/–) mice is smaller than that of control littermates. Despite the smaller size, no differences are observed in the percentage of hormone-producing cells (e. g. ACTH or GH) or progenitor cells as determined by Sox2 (not shown) or Sox9 staining (Fig. 3C). Since the inactivation of Ink4 and Cip/Kip pathways cooperate in favouring proliferation in other models, we then analyzed the proliferative potential in pituitary cells. As depicted in Fig. 3D, triple mutant *Cdk4*(R/R); *p21*(–/–); *p27*(–/–) displayed a significant increase in the ratio of Ki67-positive cells, in agreement with increased entry into the cell cycle. However, these triple

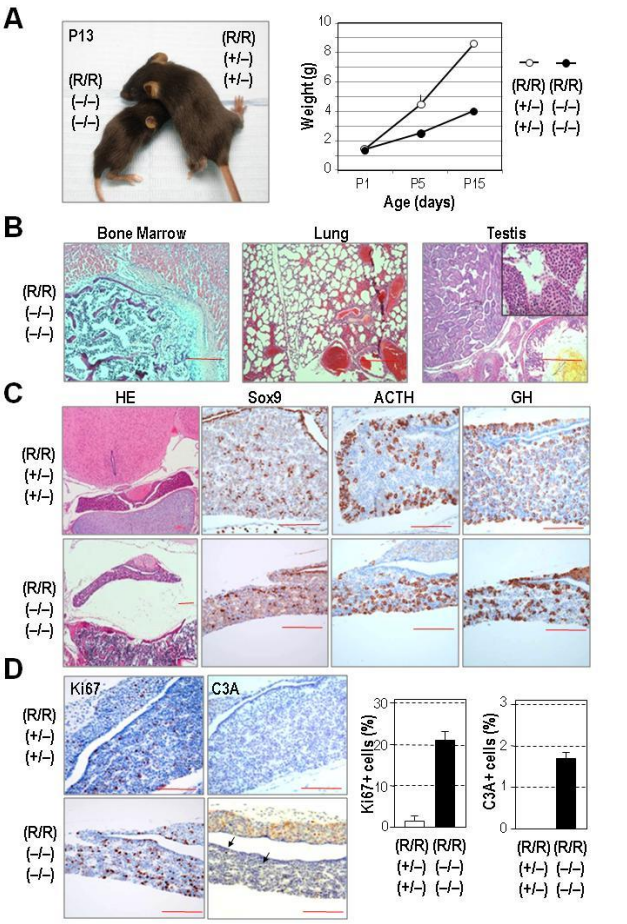

FIG 3. Triple mutant mice. (A) Representative picture of a 13-day-old triple mutant mouse compared to a littermate. Comparison of weight at different ages is shown at the right. (B) Pictures presenting massive hypoplasia of the triple mutant and characteristic organs. (C) Representative pictures pituitary gland of newborn triple mutant animals and littermate as control of H&E and the specific markers Sox9 and the hormones ACTH and Growth hormone (GH). (D) Stainings for Ki67, Caspase 3 active. Quantifications are shown at the right. Scale bar represents 100 μm.

mutant pituitaries also displayed a significant increase in the number of apoptotic cells, as detected by active caspase 3 antibodies (Fig. 3D). This phenotype is also observed in other hypoplastic tissues of our triple mutant mice (fig 3S).

Ink4 and Cip/Kip inhibitors cooperate preventing replicative stress in brain cells

In addition to the defects in the pituitary and other tissues, *Cdk4(R/R); p21(–/–); p27(–/–)* mice presented a significant hyperproliferation of the subventricular zone and the dentate gyrus in the hippocampus, not observed in any of the other genotypes. As in the case of the pituitary gland, that hyperproliferation was accompanied by induction of apoptosis in the very same regions (Fig. 4A). We have also observed in some cases a dramatic increase of DNA damage signaling (γH2AX positive cells) in the amigdala of the brain. This signal was accompanied by an induction of p53. Besides, this area is accompanied with an increase in

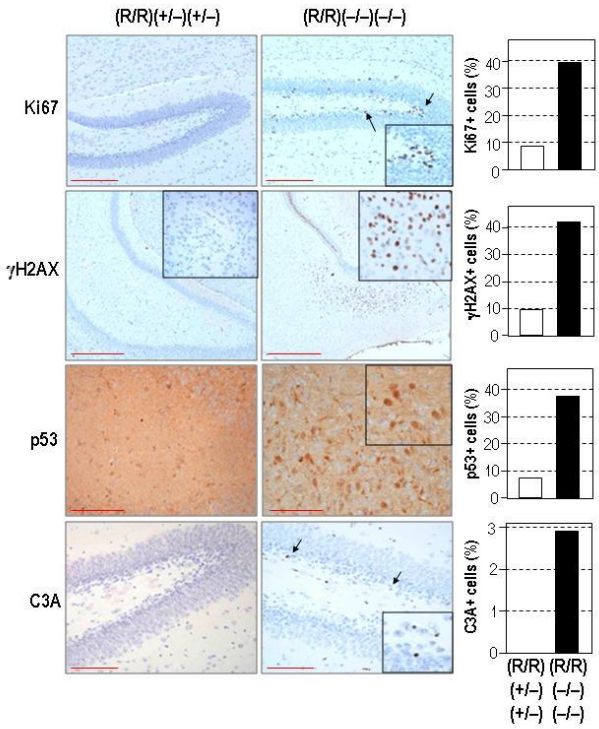

FIG. 4. Brain defects observed in newborn triple mutants. Stainings for Ki67, a proliferative marker, the DNA damage marker (γH2AX), p53 activation and Caspase 3 active, marker of apoptosis. Quantification are shown at the right of the panels. Scale bar represents 500 μm for γH2AX, 200 μm for Ki67 and Caspase 3 active and 100 μm for p53. Insert, if corresponds, represents a 4x detail of the picture.

GFAP positive cells that are a sign of reactive gliosis, which is a characteristic marker for brain injury (data not shown). The amigdala is a region of the brain involved in emotional learning and memory modulation, but also in sending impulses to the hypothalamus and other regions for the activation of the sympathetic nervous system and secretion of dopamine, norepinephrine and epinephrine. GFAP is also found increase in the hippocampus region, although in this case, the presence of p53 or γH2AX was not detected (data not shown).

The abnormalities observed within the brain and the pituitary gland correlate with the phenotypical problems observed as the smaller size and the defects in the movement and the stability of the triple mice and may explain the perinatal lethality due to brain developmental defects and/or lack of hormone signaling.

Proliferation of mutant MEFs in vitro

Triple mutant *Cdk4 R24C; p21KO; p27KO* MEFs behave as immortal and don't suffer the senesce-like state when a 3T3 immortalization protocol is perform (data not shown). These results fit with what is already published for the double mutant *Cdk4 R24C; p21KO* (Quereda et al, 2007). To address if the presence of the γH2AX that we have seen in the brain of the triple mutant mice is due to DNA damage machinery activation because of replicative stress, we performed the next experiment in triple mutant and wild-type primary MEFs. We compared the intensity of γH2AX signal in wild-type and triple mutant MEFs at different times under different concentrations of Aphidicolin, a drug that inhibits DNA pol II and a low doses cause DNA damage due to replicative stress. Triple mutants MEFs showed a higher induction of γH2AX under low doses of aphidicolin treatment (0.1 μm), suggesting defects in the induction of the DNA repair machinery (fig 4S).

Self-renewal of mutant neuroblasts in vitro

We also analyzed the relevance of these cell cycle inhibitors in self-renewal and proliferation of neuroblasts in culture. E18.5 neuroblasts were isolated from crosses between animals heterozygous for one allele and homozygous for the rest of alleles, and cultured in neurosphere-forming conditions for over a week. Primary neurosphere formation was drastically reduced in Cdk4 R24C vs. wild-type littermate neuroblasts and those differences were also found comparing *Cdk4(R/R); p21(–/–) vs the Cdk4(R/R)* alone (fig 5). The neurosphere sizes were also reduced in *Cdk4*(R/R) when compared to their wild type littermates. These differences were not significant when comparing the double mutant *Cdk4(R/R); p21(–/–) and Cdk4(R/R)* littermates (fig 5B). These results suggest a significant implication of *Cdk4* R24C in self-renewal and proliferation capacity of the neuroblasts, whereas $p21^{Cip1}$ deficiency only display minor effects. When primary neuroblasts were disgregated under papain and reseed in neurosphere-forming conditions, none or very few spheres were found in the combined genotypes, whereas wild type neurospheres were found in normal ratio (data not shown). However, further assays with new combinations of genotypes are required to fully characterize the relative roles of these alleles.

To further analyze the proliferation defects seen in our neuroblasts of mixed genotipes, primary neurospheres were treated for 1hr with 10 μm of EdU and after this, seeded in a matrigel-coated coverslip. After 15 min. the neuroblasts were completely attached to the coverslip and immunofluorescence was performed (fig 6A). Cells that are dividing within a neurosphere are as well positive for Sox9 marker, suggesting that there are at least two types of cells forming the sphere, one of them should be more undifferentiated and with proliferation capacity,

positive for Sox9. In this case, we have compared and quantify, neurospheres *p21KO; Cdk4(R/R)* and double mutants *Cdk4(R/R); p21KO*. We observed in these assays a decrease in the number of EdU positive cells in both *Cdk4 (R/R)* and *Cdk4(R/R); p21KO* mutants compared to *p21KO* cells, although the differences are not significant (fig 6B). We did not observe, however, changes in the percentage of Sox9-positive cells in neurospheres double mutant for *Cdk4 R24C* and in the double mutants *Cdk4(R/R); p21KO* compared to *p21KO* cells (fig 6C). What it is more interesting, it is the high presence of γH2AX-positive cells among the cells that are not dividing (EdU negative) suggesting that DNA damage machinery is active. γH2AX-positive cells are present also in Sox 9-negative cells (data not shown), but are more abundant in Sox9-positive cells and significantly increased in the double *Cdk4(R/R); p21KO* mutant compared to the *p21KO* (fig 6D). The staining pattern, although was mainly in a foci typical label was also found in high intense nucleus label characteristic of replicative stress signal. Consequently, if we pay attention to the DAPI staining (fig 6 A), small, pignotic, apoptotic nuclei can be found in double mutant neurospheres and a lower rate in *Cdk4 R24C* single mutant. Together, these results suggest that combination of mutations in *Cdk4*, $p21^{Cip1}$ and $p27^{Kip1}$ alleles provokes cellular stress resulting in activation of senescence pathways or apoptosis. A complete characterization of double and triple mutant neuroblasts will be required to fully understand the relative effect of the different alleles in these observations.

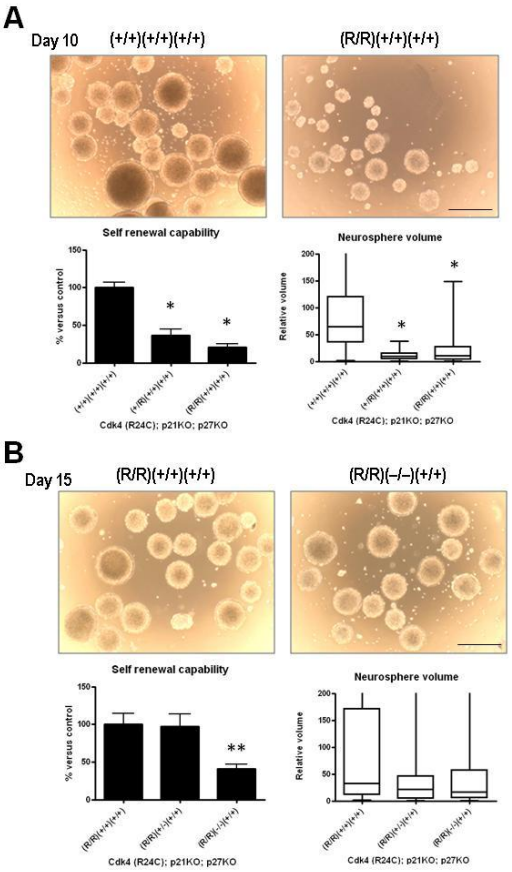

FIG 5. Self-renewal and proliferation defects in mutant neuroblasts. (**A**,**B**) Representative pictures of growing neuroblasts at different passages. Genotype indicated as *Cdk4 R24C ;p21; p27*. The quantification of self-renewal capability measured by relative number of spheres and relative volume of the neuroblasts is shown for each experiment. The volume was calculated using the formula $4/3\ \pi\ r^3$ where r is the length of the neurosphere calculated using image J software. * p<0.0001. **p<0.05

Discussion

One of the most frequent molecular events in human tumors is the deregulation of the cell cycle through Cdks hyperactivation. This may occur due to amplification, mutation or overexpression of cyclins and, more frequently, inactivation of Cdk inhibitors. Inactivation of INK4 proteins is a common oncogenic alteration in several tumor types (Malumbres & Barbacid, 2001). Similarly, $p27^{Kip1}$ and $p21^{Cip1}$ downregulation by increased proteolysis are as well frequent features of tumor cells and correlates with poor prognosis. However, lately, the implication of $p27^{Kip1}$ and $p21^{Cip1}$ in tumorigenesis is highly controversial due to opposite observations. $P27^{Kip1}$ has been found downregulated in high number of tumors, whereas it is rarely completely depleted, suggesting the necessity of basal $p27^{Kip1}$ levels for cell proliferation (Chu et al, 2008). These results togheter with the observation of an oncogenic role of the protein using animal models (Besson et al, 2007) leave the exact role of $p27^{Kip1}$ in tumorigenesis onset and manteinance as an open field. Similarly, oncogenic activities have been described for $p21^{Cip1}$ (Abbas & Dutta, 2009).

The involvement of INK4, Cip1 and Kip1 proteins in tumor development has been evaluated in vivo using different genetic mouse models. Deletion of $p21^{Cip1}$ results in altered response to DNA damage responses and increased tumor susceptibility, specifically in mesenchymal and hematopoietic cells, at advanced age (Brugarolas et al, 1995; Martin-Caballero et al, 2001). $p27^{Kip1}$ depletion in the animals causes retinal dysplasia and female infertility and animals develop adenomas in the pituitary with long latencies. Elimination of individual members of the INK4 family results in limited oncogenic phenotype, mostly restricted to reduced susceptibility to tumor development in old $p16^{INK4a}$ or $p18^{INK4c}$-*null* mice (Franklin et al, 1998; Krimpenfort et al, 2001; Latres et al, 2000; Sharpless et al, 2001; Zindy et al, 2000). The cooperation among these

families has been evaluated in different combinations. Mice deficient in $p18^{INK4c}$ in a $p21^{Cip1}$-*null* background cooperates in pituitary and lung tumors. Combined ablation of $p18^{INK4c}$ and $p27^{Kip1}$ increases the frequency of endocrine and gut tumors (Franklin et al, 2000).

Cdk4 R24C knock-in mice are a useful model since the endogenous Cdk4 protein is completely resistant to all INK4 family members in these animals. These mice develop wide-spectrum tumors at long latencies but with complete penetrance (Sotillo et al, 2001a). Mice depleted for $p27^{Kip1}$ in a Cdk4 R24C background develop pituitary tumors with complete penetrancy and very short latencies (Sotillo et al, 2001a; Sotillo et al, 2005). Ablation of *$p21^{Cip1}$* in the Cdk4 R24C background also accelerates tumor development w of tumors as single mutants with special cooperation in mesenchymal tumors (Quereda et al, 2007). In this manuscript, we now show that partial ablation of both $p21^{Cip1}$ and $p27^{Kip1}$ results in a synergistic effect in tumor development (table 1). Interestingly, in a *Cdk4 (R/R); p27KO* background, abrogation of one or two *$p21^{Cip1}$* alleles has only a minor effect in tumor susceptibility.

Perhaps more interestingly, we have observed that triple mutant mice do not develop hyperplasia but hypoplasia. Thus, a complete deregulation of these inhibitors does not result in increase tissue growth but it is likely to produce cell degeneration due to replicative stress. The progressive elimination of cell cycle inhibitors would result in inappropriate entry into S-phase or a strong shortening of gap phases resulting in a replicative stress likely to be similar to that generated by oncogenes. Thus, these triple mutant cells display a significant activation of the DNA damage machinery (γH2AX signal) and the subsequent cell death by apoptosis. The significance of these alterations in self-renewal and proliferation of different progenitor cells, such a neuroblasts, is currently a major goal of our current assays to envision the relative function of INK4 and Cip/Kip proteins in the protection against this replicative stress during development.

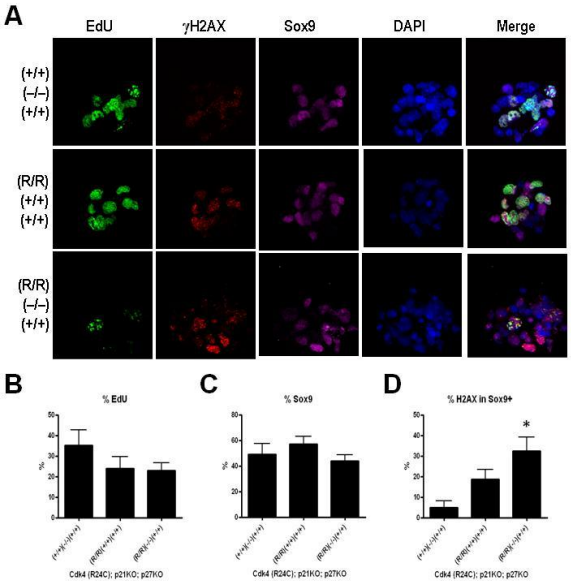

FIG 6. Elimination of cdks inhibition produces an increase in proliferative stress marked by γH2AX signal. Genotype indicated as *Cdk4 R24C ;p21; p27*. (**A**) Primary neurospheres were incubated for 1 hr with EdU and plated in a matrigel-coated coverslips for an additional 15 min. Immunofluorescences images showed for EdU (green), γH2AX (red), Sox 9 (purple) and DAPI (blue). Arrows point to examples of apoptotic cells. (**B-D**) Quantification of images for the percentage of EdU-positive cells (**B**), % Sox 9-positives cells (**C**) and % γH2AX-positive cells within the Sox 9-postives one that are negative for EdU staining. Error bars represents standard deviation from two different experiments. *p<0.05

Materials and Methods

Mice and histological analysis.

Cdk4 R24C *knock in* mice and $p21^{Cip1}$-deficient (Quereda et al, 2007) and $p27^{Kip1}$-deficient (Kiyokawa et al, 1996) animals have been reported previously. These animals were maintained in a mixed 129/Sv (25%) x CD1 (25%) x C57BL/6J (50%) background. Mice were housed at the pathogen-free animal facility of the Centro Nacional de Investigaciones Oncológicas (CNIO, Madrid) following the animal care standards of the institution. These animals were observed in a daily basis and sick mice were euthanized humanely in accordance with the Guidelines for Humane End Points for Animals used in biomedical research. Tumor latency has been considered equivalent to lifespan.

For histological observation, dissected organs were fixed in 10%-buffered formalin (Sigma) and embedded in paraffin wax. Three- or five-micrometer-thick sections were stained with hematoxylin and eosin. Additional immunohistochemical examination of the pathologies observed was performed essentially as described in (Sotillo et al, 2005). Antibodies used for paraffin sections were: glial-acidicic fibrilary protein, GFAP (1:500, DAKO, Z0334); Sox2 (1:400, Millipore, AB5603); Ki67 (Prediluted, DAKO, M7249); γH2AX (1:15000, Millipore, 05-636); p53 (1:300, Leica, NCL-p53-CM5p); Caspase 3 active (1:400, RYD, AF835); beta-TSH (1:500, NIDDK, AFP-1274789); Growth hormone (1:50, NIDDK, AFP5672099Rb); ACTH (1:750, NIDDK, AFP156102789R) and PRL (1:150, NIDDK, AFP131078Rb)

Culture of primary neuroblasts

Neuroblasts were isolated from embryonic cortex as described previously (Qian et al, 2000). Single dissociated cortical cells were cultured in uncoated 6-weel plates between 1 and 2 weeks, in serum-free DMEM with 4,5 mg/ml glucose, 10 mM L-Glutamine, 10mM Na-Pyruvate, 10 mM N-Acetyl Cysteine, N2, B27, EGF and bFGF (20 ng/ml each). For subcloning, neurospheres were collected and gently dissociated using papain (Worthington) at 37ºC for 20 min with gentle agitation. Cells were replated at equal cell density for each condition.

Immunofluorescence

Neuroblasts were incubated with EdU (invitrogen) at 10 μM for 1 hr and then plated in a coverslip coated with matrigel (BD bioscence). After 15 min in the matrigel, the spheres were attached to the coverslip. All incubations were performed at room temperature. Fixation of the spheres was done using PBS-4%PFA for 20 min. Spheres were permeabilized with PBS-Tx100 0,5% for 15 min and blocked with PBS-3%BSA-0,15%Tx100 for 30 min. EdU staining was performed following provider guideless (Invitrogen). Incubation with Sox9 (1:250, Millipore, AB5535) and γH2AX (1:750, Millipore, 05-636) was perform afterwards for 2hr. After the washing of primary antibodies with PBS-1%BSA, secondary fluorescence antibodies were incubated for 45 min. Alexa-594 goat anti-mouse and Alexa-647 goat anti-Rabbit (1:250, Invitrogen). Cells were finally stained with DAPI and mounted in prolong –anti fade with DAPI (invitrogen) and visualize using a confocal microscope SP2 (Leica).

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Supplementary Material

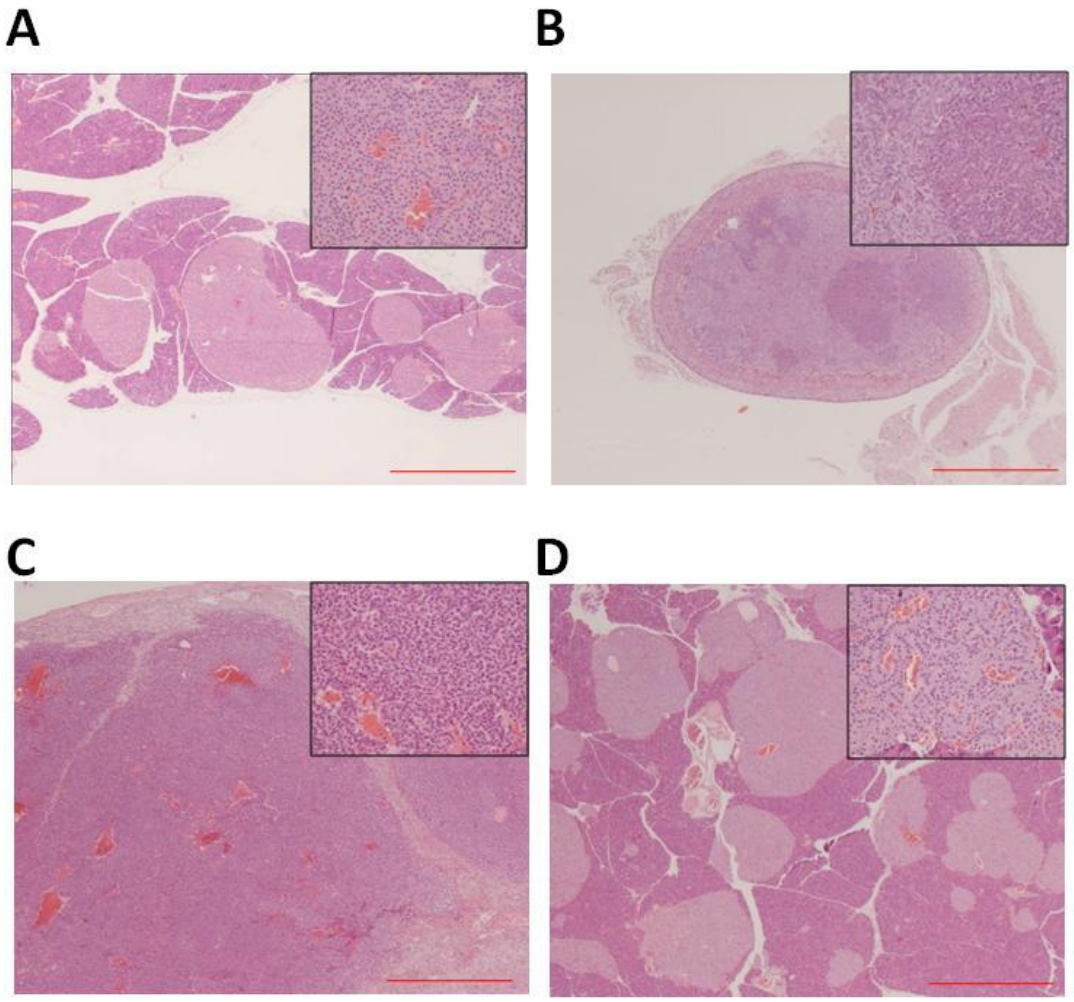

FIG 1 SUPPL. Other pathologies seen in mixed *Cdk4 R24C; p21KO; p27KO*. Microscopic images of hematoxilin-eosin stained sections are shown with a amplified detail at the upper right corner. Scale bar represents 1 mm, magnifications are a 10 X detail. (**A**) Pancreatic endocrine adenoma (**B**) Leydig cell tumor, (**C**) Pancreatic endocrine hyperplasia and (**D**) Medullary hyperplasia of the adrenal gland.

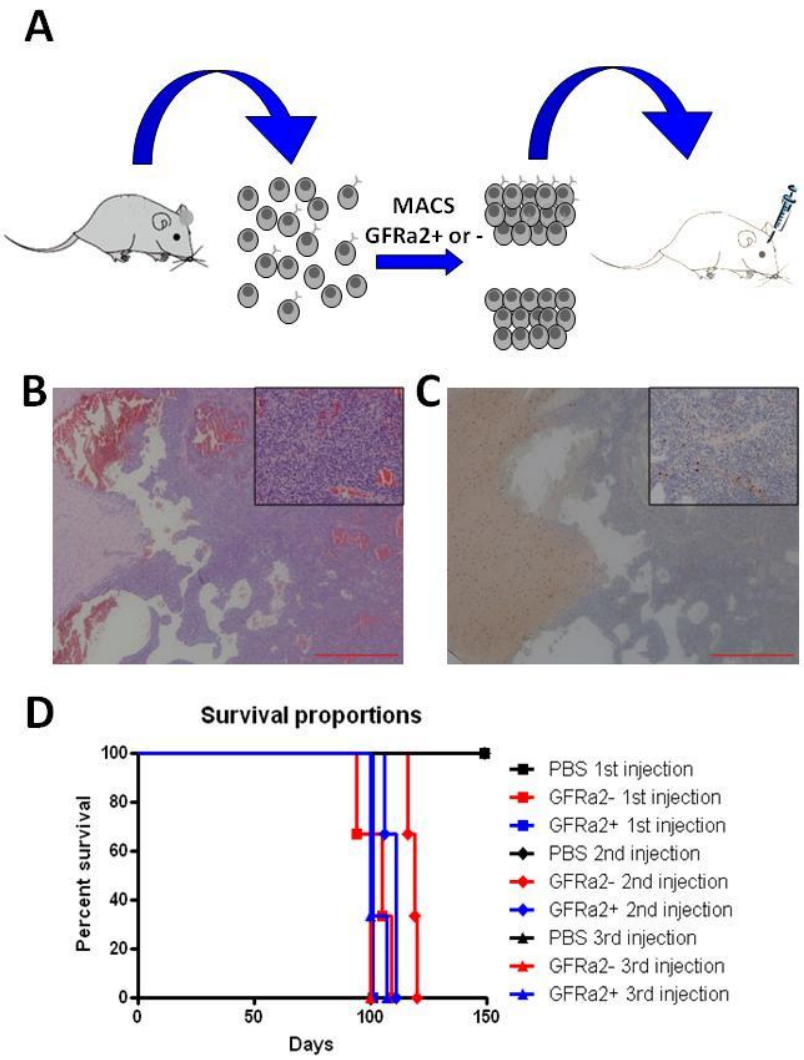

FIG 2 SUPPL. Pituitary tumor transplantation into SCID mice. (**A**) Schematic representation of the experimental procedure followed. Once the pituitary tumor were detected in mutant animals, they were sacrificed and pituitary tumoral cells were isolated and separated into GFRA2+ and – using MACS technology for being reinjected in SCID mice. Representative microscopic image of hematoxilin-eosin (**B**) or Sox 9 (**C**) stained sections of the tumors arisen are shown Scale bar represents 500 μm, magnifications are a 5 X detail of the picture. (**D**) Kaplan-Meyer representation for tumor-free survival of SCID. t=0 represents the moment of the injection.

Isolation and transplantation of GFRA2 cells

Pituitary tumors arisen from combined Cdk4 (R24C); p21; p27 mice were mince with scissors and disgregated under 0,4% collagenase type IIa (inivitrogen) for 30 min at 37ºC. Due to the fact that tumors are very hemorrhagic, erythrocytes were lysated with erythrocyte lysing buffer (NH4Cl 0,154M; KHCO3 10mM and EDTA 0,1mM; pH7,4) for 5 min at RT. Disgregated cells were incubated with GFRa2 antibody (AB5141; Chemicon) 1:50 for 1hr at 4ºC. After washing, cells were incubated with a goat-anti rabbit IgG microbeads and separation of GFRA2 positive and negative cells was perform using miniMACS columns and MACS guideline (miltenyi). Mice were anesthetized with ketamine (imalgene 500) and xilacine (rompun) and injected with the cells resuspended in PBS in a volume of less than 20ul directly in the cranial cavity using a 30G syringe.

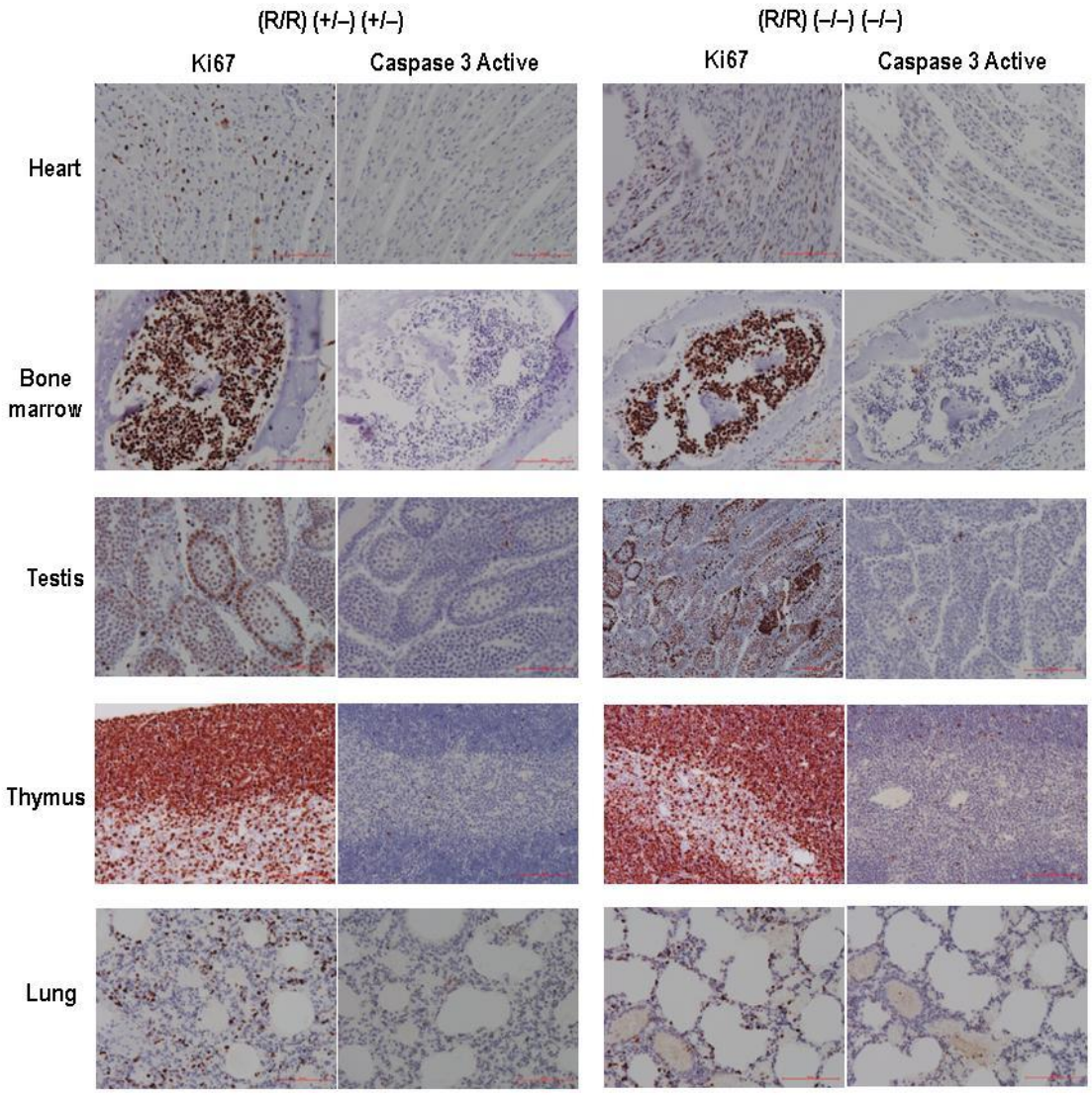

FIG 3 SUPPL. Other organs of the triple mutant present same defects as brain and pituitary gland. Ki67 and Caspase 3 active staining in the indicated organs in triple mutant animal *Cdk4 R24C;p21KO;p27KO* and the littermate used as a control. Scale bars represents 100μm.

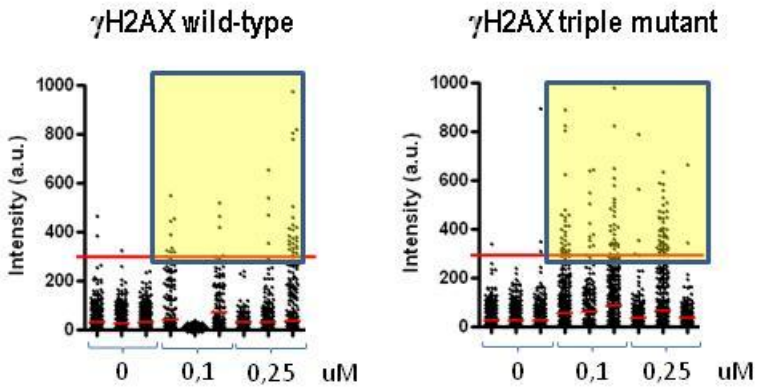

FIG 4 SUPPL. Analysis of replicative stress in MEFs. Wild-type and triple mutant *Cdk4 R24C; p21KO; p27KO* primary MEFs were treated for 8 hr with different dosages of aphidicolin. After fixation with 4%PFA, staining for γH2AX was performed and pictures were acquired using opera device. The experiment was performed in triplicates and at least two hundred cells were counted for each point. Hystogram for relative fluorescence of γH2AX signal is shown.

Mouse embryonic fibroblasts (MEFs)

Mouse embryonic fibroblasts (MEFs) were prepared from E13.5 embryos using standard protocols. Head and blood organs were removed, and the torso was minced and dispersed in 0.1% trypsin (5 min at 37°C). Cells were grown for two population doublings and then frozen. MEFs were subcultured 1:4 upon reaching confluence. MEFs cultures were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 2 mM glutamine, 1% penicillin/streptomycin and 10% foetal bovine serum (FBS).

Table 1S. Secondary pathologies in Cdk4 R24C; $p21^{Cip1}$; $p27^{Kip1}$ mutant mice

	(+/+)(+/+)(+/+)	(R/R)(+/+)(+/+)	(R/R)(-/-)(+/+)	(+/R)(+/-)(+/-)	(+/R)(-/-)(+/-)	(R/R)(+/-)(+/-)	(R/R)(-/-)(+/-)	(+/R)(+/-)(-/-)	(+/R)(-/-)(-/-)	(R/R)(+/+)(-/-)	(R/R)(+/-)(-/-)
Leydig cell hyperplasia	0,0%	14,8%	62,5%	0,0%	0,0%	30,8%	0,0%	0,0%	0,0%	100,0%	0,0%
Pancreatic hyperplasia	0,0%	36,2%	21,1%	16,7%	29,4%	72,0%	50,0%	0,0%	10,0%	57,1%	40,0%
Adrenal gland hyperplasia	0,0%	1,9%	36,8%	66,7%	5,9%	28,0%	5,0%	100,0%	40,0%	42,9%	30,0%
Myocardiopathy	0,0%	0,0%	0,0%	16,7%	5,9%	0,0%	0,0%	16,7%	0,0%	0,0%	0,0%
Hydrocephaly	0,0%	0,0%	0,0%	0,0%	0,0%	16,0%	0,0%	0,0%	0,0%	0,0%	0,0%
Lymphoid hyperplasia	0,0%	17,1%	0,0%	0,0%	0,0%	20,0%	30,0%	83,3%	40,0%	42,9%	20,0%
Atrophy spleen/thymus	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	20,0%	0,0%	30,0%
Glomerular cyst in kidney	0,0%	24,8%	10,5%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	50,0%

Table 2S. Pituitary tumor classification in Cdk4 R24C; $p21^{Cip1}$; $p27^{Kip1}$ mutant mice. PCPI. Pituitary carcinoma of the pars intermedia. PAPI. Pituitary adenoma of the pars intermedia. PHPI: Pituitary hyperplasia of the pars intermedia. PCPD: Pituitary carcinoma of the pars distalis. PAPD: Pituitary adenoma of the pars distalis. PHPD: Pituitary hyperplasia of the pars distalis. PIT: Pituitary tumor difficult to classify, with undetermined origin. PI: Pars intermedia. PD: Pars distalis

	(+/+)(+/+)(+/+)	(R/R)(+/+)(+/+)	(R/R)(-/-)(+/+)	(+/R)(+/-)(+/-)	(+/R)(-/-)(+/-)	(R/R)(+/-)(+/-)	(R/R)(-/-)(+/-)	(+/R)(+/-)(-/-)	(+/R)(-/-)(-/-)	(R/R)(+/+)(-/-)	(R/R)(+/-)(-/-)
PCPI	0,0%	0,0%	0,0%	40,0%	0,0%	20,8%	6,3%	0,0%	11,1%	0,0%	0,0%
PAPI	0,0%	2,3%	40,0%	20,0%	11,1%	41,7%	12,5%	33,3%	11,1%	0,0%	10,0%
PHPI	0,0%	7,0%	0,0%	0,0%	33,3%	16,7%	37,5%	16,7%	22,2%	28,6%	20,0%
PCPD	0,0%	7,0%	20,0%	40,0%	11,1%	25,0%	25,0%	0,0%	22,2%	28,6%	40,0%
PAPD	**0,0%**	44,2%	20,0%	20,0%	33,3%	20,8%	12,5%	50,0%	0,0%	0,0%	0,0%
PHPD	0,0%	44,2%	20,0%	0,0%	22,2%	8,3%	18,8%	16,7%	11,1%	0,0%	10,0%
PIT or PS	0,0%	0,0%	0,0%	0,0%	22,2%	25,0%	25,0%	16,7%	33,3%	71,4%	70,0%
PI and PD simultaneously	0,0%	4,7%	0,0%	20,0%	22,2%	29,2%	43,8%	16,7%	11,1%	28,6%	30,0%

4.- Discusión

La proliferación descontrolada de las células cancerígenas frecuentemente se correlaciona con una hiperactivación de las Cdks implicadas en las fases G1 y S del ciclo celular mediante amplificación, mutación o sobreexpresión de sus componentes (Cdks y Ciclinas) y, más frecuentemente, mediante la inactivación de sus inhibidores, las proteínas INK4 y Cip/Kip (Malumbres and Barbacid, 2001). Sin embargo, en los últimos años, se han descrito, tanto para $p21^{Cip1}$ como para $p27^{Kip1}$, nuevas funciones independientes de su papel inhibitorio del ciclo celular. Entre otras, ambas proteínas han sido descritas como reguladores de la auto-renovación de diferentes progenitores como los neuronales, intestinales y hematopoyéticos (Abbas and Dutta, 2009; Chu et al., 2008; Malumbres and Barbacid, 2009). Por lo tanto, bajo determinadas circunstancias, que podrían implicar la regulación de las células madre u otros papeles independientes de los descritos en la regulación del ciclo celular, $p21^{Cip1}$ y $p27^{Kip1}$ podrían tener un papel oncogénico. Para comprender el efecto conjunto de las deficiencias de los diferentes inhibidores de ciclo celular, hemos combinado el modelo Cdk4 R24C, insensible a los inhibidores INK4, con modelos que carecen de $p21^{Cip1}$ y $p27^{Kip1}$. Los ratones *knock-in* que sustituyen el gen endógeno de Cdk4 por la forma mutada Cdk4 R24C desarrollan una gran variedad de tumores y con una penetrancia completa, aunque también con una latencia alta (Sotillo et al., 2001). Por otro lado, la eliminación individual de $p27^{Kip1}$ resulta en la aparición de tumores de hipófisis con alta penetrancia, aunque también con alta latencia (Kiyokawa et al., 1996; Nakayama et al., 1996). La eliminación de $p21^{Cip1}$, en cambio, tiene un efecto muy leve en la inducción de tumores (Martin-Caballero et al., 2001). El módelo Cdk4 R24C ya ha sido utilizado para analizar el efecto de la eliminación conjunta de los inhibidores INK4 y Cip/Kip mediante el modelo que combina la eliminación de $p27^{Kip1}$ con el uso de este modelo *knock-in*. En este caso, animales dobles mutantes Cdk4(R/R); p27(–/–) desarrollaron básicamente tumores de hipófisis con latencias muy cortas (Sotillo et al., 2005).

En el trabajo presentado en esta tesis hemos podido estudiar cómo la eliminación de $p21^{Cip1}$ colabora con la ausencia de la familia INK4 (analizada mediante el uso de animales con fondo genético Cdk4 R24C) en el desarrollo de tumores de carácter mesenquimal y de

origen óseo (Quereda et al., 2007). Este efecto producido en células de tipo mesenquimal in vivo concuerda positivamente con lo observado en fibroblastos embrionarios de ratón (MEFs). En este caso, la expresión del mutante insensitivo a los inhibidores INK4, Cdk4 R24C rescata a las células deficientes para $p21^{Cip1}$ del arresto típico, similar a la senescencia que le ocurre a estas células en cultivo a lo largo de los pases. Las células dobles mutantes Cdk4(R/R); p21(–/–) se comportan como inmortales. Aunque estas células no son de por sí transformadas, son, sin embargo, muy sensibles a la transformación mediada por oncogenes como por ejemplo el oncogén Ras (Quereda et al., 2007). Por otro lado, la eliminación de $p21^{Cip1}$ en un fondo genético Cdk4(R); p27(–) tiene un efecto moderado en la latencia de los tumores. Así como la pérdida de uno o dos alelos de $p27^{Kip1}$ resulta en una reducción dramática en la vida media de los animales Cdk4 R24C, tanto heterocigotos como homocigotos, la pérdida de alelos de $p21^{Cip1}$ sólo tiene un efecto moderado. Por ejemplo, mientras la presencia de un único alelo de $p27^{Kip1}$ en un fondo genético Cdk4(R/R); p21(–/–) supone una esperanza de vida media de los animales de 30 semanas, la presencia de un alelo de $p21^{Cip1}$ en el fondo genético Cdk4(R/R); p27(–/–) sólo tiene una esperanza de vida media de 6 semanas, un incremento muy leve con respecto a la esperanza de vida media del triple mutante que es de 2 semanas (Quereda et al., 2010). A pesar de que la muerte en modelos combinados de los tres alelos, Cdk4(R); p21(–); p27 (–), es debido a un fenotipo tumoral o, en un pequeño numero de casos, al menos con presencia de hiperplasias, el triple mutante homocigoto Cdk4(R/R); p21(–/–); p27(–/–) muere perinatalmente sin que hayamos detectado presencia de tumor o hiperplasia en sus tejidos. Es más, estos animales mueren debido a una hipoplasia generalizada que provoca la incapacidad para ganar peso y la muerte principalmente por defectos neuronales y hematopoyéticos (v. Apartado 5.3). Volviendo al análisis de los genotipos intermedios, los tumores desarrollados por estos animales que combinan las tres mutaciones Cdk4(R); p21(–); p27(–) están presentes en animales simples y dobles mutantes. Sin embargo, en nuestro modelo de estudio hay una prevalencia exclusiva de tumores de tipo endocrino y angiosarcomas. Cabe destacar dentro de los tumores de carácter endocrino, el tumor de hipófisis, el más frecuente en todos los casos con

mutación de al menos un alelo de cada gen. Debido al gran número de tumores de hipófisis que se desarrollan en nuestros modelos animales, pasamos a estudiar con más detalle este tipo de tumores centrándonos en la latencia y su variedad en función de una clasificación histopatológica.

4.1.- La hipófisis como modelo tumoral para los inhibidores de ciclo celular

La hipófisis es un órgano central endocrino que regula funciones fisiológicas básicas como pueden ser el crecimiento, la reproducción o la homeostasis metabólica. La hipófisis en mamíferos se genera a partir de la bolsa de Rathke (día 9,5 de gestación en ratones). Su desarrollo termina poco después del nacimiento y no sufre cambios en la fase adulta (Zhu et al., 2007). Poco se conoce sobre la implicación de los reguladores de ciclo celular en el desarrollo de la hipófisis. Sin embargo, diversos modelos de ratón modificados genéticamente en ciertos de reguladores de ciclo sugieren a la hipófisis como una diana crítica de la desregulación del ciclo celular en el desarrollo de cáncer. Como parte del trabajo presentado en esta tesis hemos realizado una recopilación bibliográfica exhaustiva de los reguladores de ciclo celular implicados en la tumorigénesis hipofisaria tanto en humanos, como en los diferentes modelos animales (Quereda and Malumbres, 2009). El primer modelo que apareció relacionando desregulación del ciclo celular y tumorigénesis de la hipófisis fue el modelo de pRB. Los animales sin una copia de esta proteína desarrollaban tumores de pituitaria de lóbulo intermedio (Jacks et al., 1992); tumores similares aparecían en animales deficientes en la proteína $p27^{Kip1}$ (Kiyokawa et al., 1996; Nakayama et al., 1996). Los animales que portan la mutación para el alelo de Cdk4 R24C, desarrollan, sin embargo tumores en la hipófisis anterior. Los animales *knock out* para $p18^{INK4c}$ desarrollan tumores en ambos tipos celulares, la hipófisis anterior y el lóbulo intermedio. Son significativas en cuanto a latencia e incidencia, las cooperaciones que se producen combinando las deficiencias de $p18^{INK4c}$ con $p21^{Cip1}$ (Franklin et al., 1998) o $p27^{Kip1}$ (Franklin et al., 2000). Por otro lado, la deleción combinada de pRB y $p27^{Kip1}$ también coopera en el desarrollo de tumores de hipófisis (Park et al., 1999). En nuestro modelo de estudio que combina la mutación de Cdk4 R24C con la falta de las proteínas $p21^{Cip1}$ y $p27^{Kip1}$ hemos comprobado este mismo hecho. Mientras que $p27^{Kip1}$ coopera de

manera dramática en el desarrollo de tumores de hipófisis, la deleción de $p21^{Cip1}$ disminuye de manera moderada la latencia en la aparición de los tumores en un fondo genético Cdk4 R24C. Estos datos, en conjunto, sugieren la existencia de dos vías principales en la fase G1/S que son desreguladas en el desarrollo de estos tumores. Una rama estaría formada por p18/Cdk4/pRB mientras que la otra vendría dirigida principalmente por $p27^{Kip1}$ y en el que $p21^{Cip1}$ posiblemente tendría un efecto compensatorio (Figura 5). Centrándonos en el tipo de tumores que desarrollan los animales objeto de nuestro estudio podemos analizar en detalle la variación en la latencia y el tipo de tumor según vamos eliminando inhibidores de ciclo. Así, como ya hemos dicho, los animales simples homocigotos para la mutación Cdk4 R24C desarrollan tumores en la hipófisis anterior mientras que los de $p27^{Kip1}$ lo hacen en el lóbulo intermedio. La combinación de los alelos Cdk4(R); p21(–); p27(–) provoca la aparición de tumores de pituitaria en ambas localizaciones. Según se van añadiendo alelos mutados se va produciendo una disminución en la latencia y un incremento en el grado de indiferenciación del tumor. De hecho, en la combinación de alelos mutados más agresivos [Cdk4(R/R); p21(+/+); p27(–/–) y Cdk4(R/R); p21(+/–); p27(–/–)] encontramos un 70% de tumores indiferenciados en los que el tipo celular que lo originó es difícil de caracterizar. Estos tumores indiferenciados son altamente hemorrágicos y de carácter heterogéneo con regiones de células de pequeño tamaño y regiones con células grandes, tipo neurona.

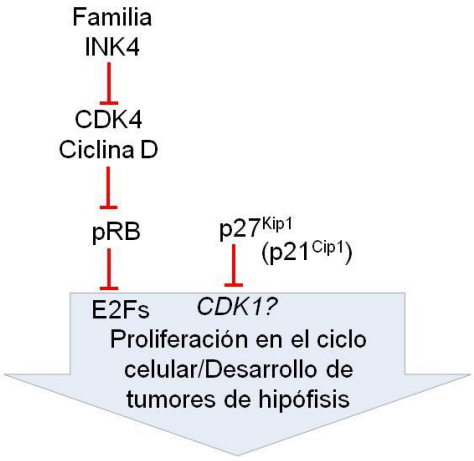

Figura 5.- Principales rutas de señalización implicadas en la regulación del ciclo celular en tumores de hipófisis

En colaboración con el grupo de investigación de la Universidad de Santiago de Compostela dirigido por Clara Alvarez hemos caracterizado una población celular específica en la denominada zona marginal de la hipófisis con características de células madre para los diferentes subtipos celulares hipofisarios (Garcia-Lavandeira et al., 2009). Estas células expresan marcadores específicos de células madre como Sox2, Sox9, SSEA4 y

Oct4, así como el marcador específico de este tipo celular y que no comparten con otras células en la hipófisis, el marcador de superficie GFRa2; además de otro característico de las células hipofisarias como el factor de transcripción Prop1. Estas células poseen los telómeros largos característicos de las células madre. Al igual que las células madre de otros tejidos, estás células son positivas para E-Caderina y b-catenina. Además, el hecho de que existan unas células positivas para vimentina cerca de estas células hace pensar en la existencia de un nicho para la homeostasis de las células madre de la hipófisis. Los esferoides crecidos *in vitro* a partir de células extraídas de la hipófisis, positivas para el marcador GFRa2, fueron capaces de diferenciar a células secretoras de hormonas y también a células similares a neuronas que presentaban marcadores específicos para este tipo celular como el isotipo III de la beta-tubulina. Curiosamente, las regiones de células de pequeño tamaño que aparecen en los tumores indiferenciados de nuestros modelos animales presentan un incremento en células positivas para los marcadores Sox2 y Sox9. Además, el porcentaje de estas células se ve incrementado conforme aumenta la agresividad del tumor y disminuye la latencia del mismo. Sin embargo, la confirmación de que estas células madre fueran las iniciadoras del tumor, no ha sido posible más allá de estos datos. Cuando se inyectaron células procedentes de tumores hipofisarios con origen incierto desde el punto de vista histopatológico, positivas o negativas para el marcador GFRa2, en ratones SCID, no hubo diferencias en el momento de aparición de los tumores. De hecho, ambos tipos celulares dieron lugar a la aparición de tumores en estos ratones SCID (Quereda et al, 2010).

4.2.- Los inhibidores de ciclo celular y el control del estrés replicativo.

Como hemos mencionado anteriormente, el triple mutante homocigoto Cdk4(R/R); p21(–/–); p27(–/–) muere perinatalmente sin presencia de tumor o hiperplasia. Estos animales nacen con la frecuencia mendeliana esperada y tienen normal apariencia en el momento de su nacimiento comparados con sus hermanos de camada. Sin embargo, a las dos semanas de vida, son claramente más pequeños que sus hermanos y presentan claros defectos en la movilidad. Este fenotipo se hace más evidente con el paso de los días y todos los animales murieron antes de la tercera semana de edad. El análisis

histopatológico de estos animales confirmó una hipoplasia generalizada con numerosos defectos en el desarrollo de los órganos. Cabe destacar la hipoplasia de la médula ósea o una disminución en el número de células de Leydig en los testículos. De manera similar a lo que ocurre con las células de Leydig, en lugar de observar hiperplasia en la hipófisis, característico del resto de animales con alelos mutados de Cdk4(R); p21(–); p27 (–), la hipófisis no era mayor en tamaño en relación con la de sus hermanos de camada, tomados como control. Por el contrario, en el caso del triple mutante la hipófisis era menor en tamaño. A pesar de este hecho, no poseía diferencias en el porcentaje de células productoras de hormonas en comparación con los controles. Sí presentaba, sin embargo, un incremento en la proliferación determinado por el número de células positivas para Ki67. Este efecto era contrarrestado por un incremento significativo en el número apoptosis que tenían lugar. Este incremento proliferativo que viene acompañado de un incremento en la muerte celular se encuentra también en otros tipos celulares como en la médula ósea y el cerebro.

De hecho, en el cerebro hemos observado que este incremento en proliferación y muerte celular viene acompañado por un incremento en el daño celular determinado por el número de células positivas para gammaH2AX. Como hemos mencionado anteriormente, los inhibidores de ciclo celular INK4 y Cip/Kip han sido relacionados con el control de la proliferación y el desarrollo del cerebro mediante la regulación de las células madre neuronales. Diferentes trabajos realizados sugieren que la presencia de proteínas como $p18^{Ink4d}$, $p27^{Kip1}$ o $p21^{Cip1}$, las cuales son necesarias para mantener el estado quiescente de las neuronas (Pechnick et al., 2008; Zindy et al., 1999). Además, la longitud de la fase G1 ha sido relacionada con el destino celular en la neurogénesis (Lange et al., 2009). En este estudio, las fases G1 más cortas dan lugar a la auto-renovación de los progenitores neuronales mientras que una fase G1 más larga provocaría la diferenciación hacia neuronas. Basándonos en los resultados obtenidos en el estudio de nuestro modelo animal triple mutante Cdk4(R); p21(–); p27(–) podemos proponer el siguiente modelo de regulación de ciclo celular por parte de los inhibidores de ciclo celular (Figura 6). En este modelo, una desregulación parcial de los inhibidores de ciclo celular conllevaría una

excesiva proliferación y como consecuencia el desarrollo tumoral. Una desregulación total, por el contrario, provocaría la degeneración y muerte celular debido al excesivo estrés replicativo que se produciría en las células. Hemos confirmado este modelo *in vitro* con células obtenidas de nuestro modelo triple mutante. De este modo, MEFs primarios triples mutantes demostraron ser más sensibles a estrés replicativo inducido por la adición de la droga afidicolina. En neurosferas obtenidas mediante la disección del cortex de animales dobles mutantes hemos podido comprobar un incremento en daño al DNA producido por estrés replicativo.

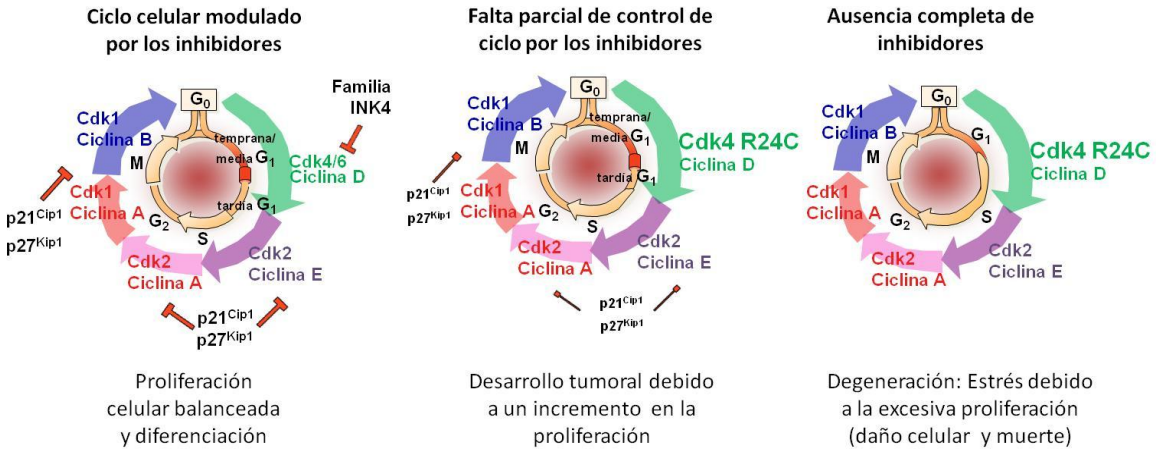

Figura 6.- Modelo propuesto para la implicación de los inhibidores de ciclo celular en el control de la proliferación. Esta hipótesis propone que una falta parcial de los inhibidores de ciclo celular conllevaría una hiperproliferación y el desarrollo tumoral. Una ausencia completa daría lugar a la muerte celular provocada por activación permanente de la maquinaria de control del daño al DNA producida por estrés replicativo.

En resumen, los resultados mostrados en esta tesis sugieren dos niveles de actuación de los inhibidores INK4 y Cip/Kip. Uno ocurre a través de la regulación de la entrada en el ciclo celular y el control de la proliferación en respuesta a antimitógenos. El otro implica una función paralela en el control del daño al DNA mediante la protección frente al estrés replicativo. Estos resultados, abren una vía de estudio para conocer en más detalle el papel de individual de cada inhibidor en la protección frente a estrés replicativo. Por otro lado, como hemos dicho, la hiperactivación de las Cdks es uno de los procesos claves que

tienen lugar en el desarrollo del cáncer. El hecho de que Cdk4 sea una proteína necesaria para el crecimiento y supervivencia de las células tumorales, pero prescindible para la viabilidad de las células normales la ha convertido durante mucho tiempo en una de las dianas favorita para el desarrollo de nuevos fármacos anti-tumorales (Malumbres and Barbacid, 2009). Sin embargo, los ensayos clínicos que han sido realizados hasta la fecha han tenido solamente un éxito muy limitado debido principalmente a la falta de especificidad y a una elevada toxicidad de los compuestos ensayados (Malumbres et al., 2008). Nuestros resultados sugieren una nueva vía conceptual donde la eliminación total de inhibición puede resultar en la muerte celular por inestabilidad genómica. Esto puede ser útil, por ejemplo, en tumores que ya de por sí tienen bajos niveles de los inhibidores, que además suelen ir relacionados con pobre prognosis. Aunque la eliminación directa de inhibidores puede ser técnicamente difícil en estos momentos, la inducción de inestabilidad genómica adicional es sin duda una estrategia que requiere nuevas investigaciones.

5.- Conclusiones

1.- La ausencia de $p21^{Cip1}$ colabora con la mutación Cdk4 R24C en la formación de tumores mesenquimales y endocrinos in vivo.

2.- La expresión la forma mutante Cdk4 R24C rescata a los fibroblastos embrionarios de ratón deficientes para $p21^{Cip1}$ de la senescencia celular.

3.- Los animales con diferentes combinaciones de los alelos Cdk4(R); p21(–); p27(–) mueren debido a la aparición de tumores fundamentalmente de carácter endocrino, con variaciones en las latencias dependiendo de la agresividad del genotipo.

4.- La eliminación de $p21^{Cip1}$ sólo disminuye de manera moderada la latencia de los tumores en animales de fondo genético Cdk4(R); p27(–).

5.- Los inhibidores de ciclo celular INK4 y Cip/Kip son claves en la inhibición de la tumorigénesis de la hipófisis.

6.- El triple mutante homocigoto Cdk4(R/R); p21(–/–); p27(–/–) muere de manera perinatal debido a una hipoplasia generalizada presentando sus tejidos un exceso de proliferación y de muerte por apoptosis. Ello sugiere que los inhibidores de Cdks son necesarios no sólo para frenar el ciclo celular en respuesta a señales antimitógenicas sino también para proteger a las células de la inestabilidad genómica y del estrés replicativo inducido por el exceso en señales proliferativas.

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